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INTERFERENCE WITH ANTITOXIC RESPONSES IN IMMUNISATION WITH COMBINED PROPHYLACTICS.

MOLLIE BARR AND MONA LLEWELLYN-JONES

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In a previous paper (Barr and Llewellyn-Jones, 1953) we recorded experiments showing that interference with the response of animals to one component of a combined prophylactic occurred in certain circumstances In a single experiment, two groups of guinea-pigs received an injection of 0·05 ml. of T.A.B. vaccine (typhoid, paratyphoid A and B); 28 days later they received a dose of 0·05 ml. of a mixture of T.A.B. vaccine and tetanus toxoid (T.A.B.T.), which was repeated after a further 28 days. The second group received only two doses of 0·05 ml. of T.A.B.T. separated by an interval of 28 days. The tetanus antitoxin titres of blood samples taken 10 days after the second injection of T.A.B.T. were higher and less scattered in the second group than in the first.

In another experiment it was shown that guinea-pigs previously immunised with diphtheria toxoid had significantly lower tetanus antitoxin titres after immunisation with two doses of any of three combined diphtheria-tetanus prophylactics than normal guinea-pigs of the same weight. In an attempt to throw some light on the mechanism of the interference, caused apparently by the preliminary injections either of T.A.B. vaccine or diphtheria toxoid, we have carried out further experiments. This interference is a matter of practical importance. It is possible that in attempts to immunise against two or more diseases, two injections of a combined prophylactic might be given, and an additional dose of one component only, if the two doses were considered to produce insufficient immunity to this component. The spacing of such an extra dose, in relation to that of the injections of the combined prophylactic, might strongly influence the degree of immunity finally produced by both components of the preparation.

EXPERIMENTAL.

The Effect of a Preliminary Injection of T.A.B. Vaccine on the Response to Immunisation with Tetanus Prophylactics.

The following materials were used:

(*a*) T.A.B. vaccine containing 10^9 *S. typhi*, 5×10^8 each *S. paratyphi* A and B per ml., 0·5 per cent phenol.
(*b*) Crude formol tetanus toxoid, 9 Lf/ml., 0·5 per cent phenol.
(*c*) T.A.B.T., which was a mixture of the vaccine (*a*) and the toxoid (*b*).

The vaccine was added as a concentrated suspension, so that in the final mixture the toxoid was diluted about 5 per cent.

All injections were of 0·05 ml. diluted in saline to 1 ml. and administered subcutaneously. Eight groups of guinea-pigs of approximately equal weight were injected with one or more of these prophylactics given in different order. The scheme of injection is set out in Table 1.

TABLE I.—*The Tetanus Antitoxin Titres of Guinea-pigs after Immunisation with Two Doses of Tetanus Prophylactics.*

Group.	1st inj.	2nd inj.	Number of guinea-pigs with titres (units/ml.)— Over / Under	.. 0·01.	0·01. 0·02.	0·02. 0·05.	0·05. 0·1.	0·1. 0·2.	0·2. 0·5.	0·5. 1·0.	1·0. 2·0.	2·0. 5·0.	5·0. 10.	10. 20.	20. 50.	Geometric mean titre.
1	Toxoid	Toxoid		–	–	–	–	–	–	1	2	5	9	6	3	6·8
2	,,	T.A.B.T.		–	–	–	–	–	–	–	–	4	2	4	2	8·7
3	T.A.B.T.	Toxoid		–	–	–	–	–	–	–	1	2	5	4	1	7·7
4	,,	T.A.B.T.		–	–	–	–	–	–	–	1	9	8	4	6	7·9
5	Toxoid	Toxoid		–	–	–	–	–	–	–	3	3	6	3	–	5·0
6	,,	T.A.B.T.		–	–	–	–	–	–	–	2	3	7	2	1	5·9
7	T.A.B.T.	Toxoid		1	0	0	0	2	0	1	3	4	2	–	–	<1·1
8	,,	T.A.B.T.		1	0	0	0	1	1	1	0	7	3	–	–	<1·5

Groups 5–8 had received an injection of T.A.B. vaccine 28 days before immunisation with tetanus prophylactics.

Groups 1 to 4 received two injections of 0·05 ml. of tetanus toxoid and/or T.A.B.T.; groups 5 to 8 received the same materials preceded by an injection of 0·05 ml. of T.A.B. vaccine. The interval between injections was in all cases 28 days. The guinea-pigs were bled 10 days after the last of the series of injections and the tetanus antitoxin content of the serum of each animal titrated by the mouse method (Glenny and Stevens, 1938). Titrations were made at approximately two-fold differences and the results are shown in Table 1. Geometric means were calculated, taking each titre as the geometric mean of the limits within which it lay.

There is no significant difference between the responses of groups 1 to 6, but it is evident that groups 7 and 8 gave an undoubtedly poorer response. This is shown by the lower geometric mean titre, and what is more significant, the greater scatter of values. It is clear that the poor response is associated, not with the effect of the previous injection of T.A.B. vaccine alone, but with the use of a primary stimulus for tetanus containing T.A.B. vaccine, for groups 5 and 6 (which received the preliminary T.A.B. injection) gave substantially the same response as groups 1 to 4, which received no preliminary injection.

The Effect of Pre-existing Diphtheria Immunity on the Response to Immunisation with Tetanus Prophylactics.

The materials used were :

1. Purified tetanus toxoid completely adsorbed on a mineral carrier, and containing 6 Lf/ml.
2. Combined diphtheria-tetanus toxoid similarly adsorbed on the same amount of carrier, and containing 60 Lf purified diphtheria toxoid and 6 Lf purified tetanus toxoid/ml.

The dose injected was 0·005 ml., and the interval between the two injections was 28 days. The guinea-pigs were bled 10 days after the second injection of tetanus or combined prophylactic. Two large groups of guinea-pigs were used. One consisted of animals immunised with two injections of diphtheria prophylactic some 6 months previously, the other of normal animals of approximately the same weight. These groups were further subdivided and injected as shown in Table II.

The results resemble those in Table I. When the primary stimulus to tetanus contained diphtheria toxoid, a much poorer response to tetanus toxoid was shown

TABLE II.—*The Tetanus Antitoxin Titres of Normal and Diphtheria-Immune Guinea-pigs after Immunisation with Two Doses of Prophylactic containing Tetanus Toxoid.*

Guinea-pigs.	1st inj.	2nd inj.	Number of guinea-pigs with tetanus antitoxin titres (units/ml.)— Over / Under .. 0·01.	0·01. 0·02.	0·02. 0·05.	0·05. 0·1.	0·1. 0·2.	0·2. 0·5.	0·5. 1·0.	1·0. 2·0.	2·0. 5·0.	5·0. 10.	10. 20.	20. 50.
Normal	D + T	D + T	–	–	–	–	–	–	1	3	6	2	3	–
Diphtheria-immune	D + T	D + T	2	1	0	1	2	0	1	6	1	1	–	–
Normal	D + T	T	–	–	–	–	–	–	–	1	2	3	0	1
Diphtheria-immune	D + T	T	2	1	0	1	1	2	3	2	1	–	–	–
Normal	T	D + T	–	–	1	1	0	0	1	2	3	3	0	1
Diphtheria-immune	T	D + T	–	–	2	0	0	0	1	1	5	4	1	–

D + T = combined diphtheria-tetanus prophylactic.
T = tetanus prophylactic.

by guinea-pigs previously immunised with diphtheria toxoid than by normal guinea-pigs. It is, however, of interest that both groups injected with tetanus followed by combined prophylactic contained two poor responders, while the remaining animals had titres distributed over the same levels as the normal control groups above them in Table II. It is possible that this result could be accounted for by the weight of these guinea-pigs.

The average weight of these two groups of animals was 785 g., compared with 575 g. for those that received two injections of combined prophylactic and 675 g. for those injected with combined prophylactic followed by tetanus alone. The dosage injected, in terms of body weight, was therefore somewhat lower for the groups injected with tetanus prophylactic followed by the combined preparation. It is, however, possible that the presence of diphtheria toxoid in the first dose of prophylactic injected into the other four groups of guinea-pigs exerted a synergic effect upon the tetanus response, though when this prophylactic was injected in the same dosage into guinea-pigs of more normal weght (250–400 g.) no synergic effect was observed. The results of this test are shown in Table III.

TABLE III.—*The Tetanus Antitoxin Titres of Guinea-pigs* (250–400 *g.*) *after Immunisation with Two Doses of Tetanus Prophylactic and Diphtheria-Tetanus Prophylactic both containing* 6 *Lf/ml. Tetanus Toxoid.*

Prophylactic	Number of guinea-pigs with tetanus antitoxin titres (units/ml.)—				Geometric mean titre.
	Over 1.	2.	5.	10.	
	Under 2.	5.	10.	20.	
Tetanus	2	4	4	4	3·73
Diphtheria-tetanus	2	5	5	3	3·41

When the dosage in terms of body-weight was reduced, *i.e.*, the same dose injected into animals of about double the weight, a synergic effect might have been obtained. We have already shown that although the addition of a T.A.B. vaccine improved the response of guinea-pigs to immunisation with tetanus toxoid at one level of dosage, no synergic effect occurred when the dosage of the same combined preparation was increased (Barr and Llewellyn-Jones, 1953). It appears probable that the somewhat poor response of these animals in the last two groups of Table II was due to the effect of weight, for the general distribution of titres of the remaining animals is not typical of that shown by groups in which interference has occurred. In such groups the distribution tends to be more irregular and includes some very poor responders, while the peak value is lower than that of the controls.

Some groups of animals in which interference with a response had occurred were bled and tested again 28 days after the second injection in order to determine whether the response had been delayed. It was found that those guinea-pigs which had produced antitoxin by the 10th day after the second injection had given secondary responses, because a fall in titre occurred thereafter. It would therefore appear that the magnitude, not the speed, of the response had been reduced by interference.

It is clear that the interference with the tetanus response shown in Tables I and II must have been brought about by a secondary response to the T.A.B. vaccine or diphtheria toxoid present in the first injection of combined prophylactic. Interference with the development of potential immunity to tetanus toxoid might have occurred through crowding out of the immunity-producing mechanism by a speedy secondary response to the other component. Alternatively or in addition this response might still have been actively occurring at the time of the second injection of tetanus toxoid or combined prophylactic: this might have reduced the secondary response to the tetanus antigen. Thus interference may have occurred with the first or the second or both injections of the tetanus toxoid component, as a result of the superior speed or the prolonged effect of the secondary response to the other antigen present in the first dose of combined prophylactic. In view of the fact that in our earlier experiment (Barr and Llewellyn-Jones, 1953) tetanus antitoxin production was better in diphtheria-immune than in normal animals after the *first* injection of combined diphtheria-tetanus toxoid, it is perhaps more reasonable to believe that most if not all of the interference was with the second injection. It cannot however be assumed that antitoxin production after primary stimulation constitutes a measure of the degree of potential immunity established.

The Effect of an Injection of Vaccine between Two Injections of Combined Prophylactic.

This experiment was concerned with the effect on tetanus antitoxin production of an injection of T.A.B. vaccine given between two injections of T.A.B.T. The batches of these antigens were the same as those used in the first experiment, in which the additional injection of vaccine was given before the course of combined immunisation.

Four groups of guinea-pigs of comparable weight were used. Two of these groups received two injections, each of 0·02 ml. T.A.B.T., separated by an interval of 6 weeks, and the other two had doses of 0·05 ml. given at the same interval. One group at each level of dosage received in addition an injection of 0·02 or 0·05 ml. T.A.B. vaccine 4 weeks after the first injection. All the animals were bled 10 days after the second injection of T.A.B.T. and their tetanus antitoxin titres are shown in Table IV.

In both series the intermediate injection of vaccine had a beneficial effect on the response, although at the higher level of dosage the difference between the geometric means of the titres for the two groups is not significant (the t-test applied to the logarithms of the titres gives $0{\cdot}05 < P < 0{\cdot}10$). The scatter of titres shown by the control group 1, injected with doses of 0·02 ml., was considerably greater than that of group 2, which received an additional dose of vaccine

TABLE IV.—*Tetanus Antitoxin Titres of Guinea-pigs after Immunisation with Two Doses of T.A.B.T. given at an Interval of 6 Weeks.*

Group.	Dose.	Number of guinea-pigs with titres (units/ml.)— Over / Under	0·02. / 0·05.	0·05. / 0·1.	0·1. / 0·2.	0·2. / 0·5.	0·5. / 1·0.	1·0. / 2·0.	2·0. / 5·0.	5·0. / 10.	10. / 20.	20. / 50.	Geometric mean titre.	t.	P.
1	0·02		2	1	1	0	0	4	2	1	0	1	0·818	2·249	0·02–0·05
2	0·02		–	–	–	–	1	2	6	3	2	–	3·73		
3	0·05		–	–	–	–	–	–	3	5	4	–	7·29	1·893	0·05–0·10
4	0·05		–	–	–	–	–	–	–	6	4	2	11·44		

Groups 2 and 4 received in addition an injection of T.A.B. vaccine 4 weeks after the first injection of T.A.B.T.

only; there is a difference between the geometric means for the two groups. It is possible that this was due to some neutralisation of the T.A.B. antigen in the second dose of T.A.B.T. by circulating antibodies produced in response to the injection of vaccine alone, given 14 days earlier. It is probable that this second dose of T.A.B.T. was given at a time when the titre of such antibodies was at its height. If this were the case, groups 2 and 4 in Table IV would receive in effect a dose of combined prophylactic in which the tetanus toxoid was in considerable excess, while groups 1 and 3, having had no injection of either component for 6 weeks, received a dose of a well-balanced mixture. If this explanation is correct, it is possible that the T.A.B. component of the second dose of combined prophylactic may have had little antigenic effect, and that the synergic effect on one component may have occurred at the expense of the response to the other. If the time interval between the injection of vaccine and the second injection of combined prophylactic had been longer a different result might have followed, for the titre of circulating antibodies to the T.A.B. vaccine might have fallen.

DISCUSSION.

It is evident from the results presented in Tables I and II that interference with the development of immunity to tetanus toxoid may occur in animals immunised with two injections of a combined prophylactic containing another antigen to which they have some pre-existing immunity. Similar interference occurred when the first injection was of the combined prophylactic and the second injection of tetanus toxoid, but not when the order of these injections was reversed.

Thus it would appear that if a primary injection of one antigen has already been given, satisfactory reinforcement of the immunity to it and primary immunisation with another antigen can be brought about by giving one injection of the new antigen alone followed later by a dose of combined prophylactic. If an adequate dose of the new antigen is used interference with the response would not be expected to occur.

It is probably not generally realised that the antigenic effect of heterologous antitoxic protein can interfere with the development of immunity to toxoid in combined active and passive immunisation : such interference is distinct from, though it may be additional to, any that may occur through partial neutralisation of the toxoid by its specific antitoxin.

One of us (M.B.) was concerned in an investigation of methods for the protection of army personnel against tetanus. It was found that the response of previously unimmunised men to two injections of tetanus toxoid was reduced if they received a dose of 500 units of tetanus antitoxin at the same time as the first injection of toxoid, but in a different site. This result has already been reported briefly (Sachs, 1952). A comparison of the distribution of the titres of these men with those of a control group who had no injection of antitoxin suggested that some interference was due to a reduction in the effective primary stimulus through partial neutralisation of the toxoid by antitoxin. Some of the active-passive group, however, showed accelerated elimination of the injected antitoxin, and their response to the tetanus toxoid was poorer than that of men who showed a normal rate of elimination. It was therefore suggested that precipitin formation had brought about interference with the response of these men to immunisation with tetanus toxoid, and indeed some of those who eliminated the antitoxin with abnormal rapidity were thought to have had a previous injection of horse serum, and might therefore have responded rapidly to the injection given in combined active and passive immunisation. The full results of this work will be the subject of a joint report to be published by the War Office. It was this finding that led us to do the experiments on interference in combined prophylaxis, for it suggested that one component of a combined antigen might confer little benefit on persons who had little or no basal immunity to it, but well-developed basal immunity to another component of the antigen. There appears to be a similarity between the results of this active-passive work and of the experiments on combined prophylactics that we have recorded here. Interference with primary immunisation with toxoid occurred by preferential response to another antigen administered simultaneously with the first injection.

Mention has also been made (Sachs, 1952; Barr and Sachs, 1952) of interference (due to precipitin formation) with the response of an actively immunised man to

a boosting dose of tetanus toxoid given at the same time as an injection of 500 units of tetanus antitoxin. This man had had an earlier injection of horse serum, and thus had immunity to both tetanus toxoid and horse protein.

An animal with good potential immunity to an antigen A and poor potential immunity to another antigen B might respond sufficiently rapidly to A after an injection of A + B, for interference with antibody formation to B to occur. If, however, the immunity to both components was of a high order, and there was a considerable amount of circulating antibody to A and no antibody but good potential immunity to B, a reversed result might conceivably occur because of neutralisation of antigen A by circulating antibody after injection. It is therefore possible that the presence or absence of circulating antibody to one component might play a considerable part in determining whether an increased or decreased response to another component would occur after an injection of both antigens. The man referred to by Barr and Sachs had a high titre of actively produced tetanus antitoxin at the time of the injections of tetanus toxoid and antitoxin given in combined active and passive immunisation. He probably, however, had no circulating precipitin, as he had had no injection of horse antitoxic protein for some years. Neutralisation of some of the toxoid by circulating antitoxin, thus reducing the effective dose of tetanus toxoid, may have facilitated a preferential secondary response to the dose of horse protein administered at the same time.

It is reasonable to suppose that both dosage and spacing of injections may affect the responses in combined prophylaxis. In an unfinished experiment we have found that a preliminary injection of *H. pertussis* vaccine may bring about interference with the diphtheria response to immunisation with two doses of a combined alum-precipitated diphtheria-pertussis prophylactic at a certain level of dosage. When the dosage of both antigens is increased considerably, no interference occurs. The interference at low dosage may be due to crowding-out by the secondary response to the vaccine contained in the first dose of combined prophylactic. The absence of interference at high dosage might be due to a reduction in the effect of the vaccine in this dose through neutralisation by very large amounts of circulating antibody produced in primary stimulation, or to the dosage of diphtheria A.P.T. being sufficient to overcome the crowding-out by the pertussis responses.

If the interference with response to tetanus toxoid, shown in our experiments, was due to reduction of the secondary response to the second dose by prolonged immunological activity connected with the secondary response to another antigen in the first dose of combined prophylactic, this interference might be reduced or abolished by delaying the second dose of toxoid. More work is needed on the spacing of injections and on the application of these considerations to human prophylaxis.

It is perhaps a matter of interest that a secondary response to T.A.B. vaccine, to horse antitoxic protein and to diphtheria toxoid, can all interfere with immunisation with tetanus toxoid. Little is known of the character of the responses of guinea-pigs to subcutaneous injections of bacterial vaccines, or indeed of the nature and number of antibodies produced thereby. It is known, however, that certain antibacterial bodies developed in horses after intravenous injections of cultures such as pneumococci are not associated with the same serum protein as the antitoxins. It would appear that the production of one particular kind

of antibody may interfere with the production of another, even though these two antibodies may represent modifications of different serum proteins.

SUMMARY.

Interference with the development of immunity to tetanus toxoid occurred in animals immunised with two injections of a combined prophylactic containing another antigen to which they had some immunity Similar interference occurred when the first injection was of the combined prophylactic and the second injection of tetanus toxoid alone, but not when the order of these injections was reversed.

This interference was due to the secondary response to the other component of the combined prophylactic, and occurred whether this was T.A.B. vaccine or diphtheria toxoid.

An injection of vaccine given 14 days before the second of two injections of a combined prophylactic containing the same vaccine resulted in an increased response to the toxoid component of the prophylactic.

We are indebted to Brigadier A. Sachs, C.B.E., M.D., for permission to refer to the results of combined active and passive immunisation of man. We should like to thank Mr. P. A. Young for statistical work on Table IV, and Mr. A. T. Glenny, F.R.S., for his help in discussing the results.

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THE PREPARATION OF NON-FLOCCULATING DIPHTHERIA ANTITOXIN BY ABSORPTION OF NON-ANTITOXIC ANTIBODIES.

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THE isolation of diphtheria antitoxin with a purity of 211,000 units per gramme of protein was reported by Pope and Stevens (1953). This material was obtained after purification based on the removal of non-antitoxic antibodies from the starting commercial peptic antitoxin. This value for the purity of the product was considerably higher than those reported previously. Pope and Healey (1939) found 135,000 u./g. protein and Northrop (1942) described a crystalline preparation with 130–140,000 u./g. protein. It was considered advisable to examine this new preparation to determine whether it was homogeneous on immunological grounds. It was assumed that homogeneous diphtheria antitoxin would show the same flocculation behaviour with a series of toxin fractions of widely differing purities in terms of Lf units per mg. of protein nitrogen, and that marked differences in flocculation characteristics would indicate that the antitoxin was still heterogeneous, being contaminated with non-antitoxic antibodies.

The purpose of this paper is to describe the results obtained from an examination of this antitoxin, and of others in which the absorption of non-antitoxic antibodies has been carried still further.

MATERIALS.

Antitoxin.—The highly purified antitoxin, MS 453(3), with a purity of 211,000 units/g. protein was that described by Pope and Stevens (1953).

The other purified antitoxins prepared by absorption of non-antitoxic antibodies are described in detail later.

Toxin.—Culture filtrates from *C. diphtheriae* (PW. 8 strain) grown on tryptic digest medium (Linggood and Fenton, 1947) were ultrafiltered to concentrate the toxin to an Lf value of 2000 units or more per ml. Dilutions were then prepared with a value of 1000 Lf u./ml., and the toxin separated fractionally by the addition either of solid sodium citrate or a mixture of solid potassium phosphates. Details of these preparations are given in Tables I and II.

METHODS.

The optical density method described by Pope and Stevens (1953) was used to study the interaction between the purified antitoxin and the toxin fractions.

Antitoxins and toxins were assayed, *in vitro* by the flocculation test and *in vivo* by intradermal injection into guinea-pigs.

Preparation of toxin fractions.

For the purpose of this work it was desirable to have a number of toxin fractions which differed widely in their purities in terms of Lf units associated with either the total nitrogen or protein nitrogen per ml. It has been shown by Pope, Stevens, Caspary and Fenton (1951) that the crude culture filtrates of *C. diphtheriae* (PW. 8) contain a large number of antigens, and it was expected that the relative distribution of these antigens would differ in fractions of differing purity. The preliminary concentration of the toxin to 1000 Lf u./ml. by ultrafiltration and the use of solid non-nitrogenous precipitants made the separation of fractions a simple procedure. Details of two fractionations of this kind are shown in Tables I and II.

RESULTS.

In Table I the results obtained using solid trisodium citrate as the precipitant are given. Each fraction after precipitation by the concentration of citrate shown

TABLE I.—*The Fractionation of* 1000 *Lf Units/ml. Ultrafiltered Crude Diphtheria Toxin with Solid Trisodium Citrate.*

Toxin fraction.	Citrate concentration.	Per cent. Lf units.	Lf/mg. P.N.*
MS 460 (1)	0 to 20 per cent	6·0	370
„ (3)	30 „ 35 „	4·0	208
„ (4)	35 „ 40 „	16·0	790
„ (5)	40 „ 45 „	45·0	1685
„ (6)	45 „ 50 „	15·0	1163
Total recovery		86·0	

* Determined by precipitation and washing the precipitate with 5·0 per cent trichloracetic acid.
MS 460 (2) lost accidentally.

was separated by filtration with a little filter-aid (Hyflo Supercel) and the precipitate dissolved in water. The fractions so obtained showed considerable variation in terms of purity expressed as Lf u./mg. protein N. Unfortunately one fraction (MS 460/2) was lost by accident.

Another batch of toxin, grown under somewhat different cultural conditions, was concentrated to 1000 Lf u./ml. and then fractionated with a solid phosphate mixture with the results shown in Table II. Three fractions were obtained, all with comparitively high purity figures in terms of either Lf u./mg. total or protein N.

TABLE II.—*The fractionation of* 1000 *Lf Units/ml. Ultrafiltered Crude Diphtheria Toxin with a Solid Potassium Phosphate Mixture at pH* 7·4.

Toxin fraction.	Phosphate* concentration.	Per cent Lf units.	Lf/mg. T.N.	Lf/mg. P.N.†
MS 464 (2)	30–35 per cent	11·0	1815	2060
„ (3)	35–40 „	67·0	2320	2440
„ (4)	40–45 „	12·0	2040	2140
Total recovery		90·0		

* The solid phosphate mixture had the following composition: K_2HPO_4 83·3 per cent, KH_2PO_4 16·7 per cent (finely ground to an intimate powder).
† Determined by precipitation and washing the precipitate with 5·0 per cent trichloracetic acid.

The reaction of the toxin fractions with antitoxin.

Each toxin fraction prepared as described was tested for its Lf value and a dilution prepared from it having substantially 100 Lf u./ml. Preparation of the toxin-antitoxin mixtures and the measurement of the optical density were carried out as already described by Pope and Stevens (1953). Both the original peptic antitoxin (purity 16,000 u./g. protein) and the material prepared from it by absorbing non-antitoxin antibodies (purity 211,000 u./g. protein) were tested against these toxin fractions.

It is not possible to reproduce here all the curves obtained and the results are therefore given in a summarised form.

No two toxin fractions gave the same flocculation curves against either the original peptic antitoxin or the highly purified antitoxin prepared from it. With any toxin fraction the original peptic antitoxin always gave a larger floccule precipitate than did the purified antitoxin.

All the curves obtained from this series of toxin fractions and either antitoxin were irregular in shape and similar to those previously described. Two of the citrate-toxin fractions, MS 460(3) and MS 460(6), and one of the phosphate-toxin fractions, MS 464(3), gave very clear double-zone flocculation in the range 0 to 240 per cent toxin/antitoxin when tested against the highly purified antitoxin ; against the original peptic antitoxin they showed a single flocculation only. This point is illustrated in Fig. 1. where the flocculation curves for the citrate fraction MS 460(6) are shown against the two antitoxins, MS 453(3) and DP 1252. Incidentally it may be pointed out that this toxin fraction, when tested against the crude peptic antitoxin DP 1252, but not against the highly purified antitoxin MS 453(3), gave the closest approach to a curve of the type reported by Pappenheimer and Robinson (1937), which we have obtained in our work. It can only be concluded that, notwithstanding its exceptionally high purity, this antitoxin is not homogeneous in an immunological sense, based on the markedly varying flocculation density curves given by these toxin fractions against the highly purified antitoxin. This heterogeneity in antibody composition is even more clearly shown by certain of the toxin fractions which produce with it two zones of flocculation. The presence of two distinct flocculations is clear indication that at least two antigen-antibody systems are involved in this series of toxin-antitoxin mixtures, and these results suggested that further removal of non-antitoxic antibodies by absorption methods should be possible.

Results Obtained after Exhaustive Absorption of Antitoxin.

We have recently reported that antitoxin obtained from a horse after immunization with " flash-heated toxoid " could be isolated by ammonium sulphate fractionation and then purified by absorption methods so that it gave a symmetrical flocculation curve with crude diphtheria toxin. The same antitoxin isolated by ammonium sulphate fractionation was also submitted to purification by pepsin treatment (Pope, 1939) and then to purification by absorption of other antibodies, which was carried out in the following manner :

Concentrated peptic antitoxin 100 ml. (1950 u./ml.), was mixed with 100 ml. water and 200 ml. of a suspension of washed *C. diphtheriae* (as a moderately thick cream prepared from *C. diphtheriae* grown for 3 days). This mixture was ball-milled for 3 days with the addition of 20 g. sharp sand and 5·0 ml. toluol. At

the end of the grinding the contents of the ball-mill were washed out to give a volume of 1·0 l. After adding filter-aid the suspension was filtered on a Buchner funnel and the cake washed with water to yield 1·0 l. filtrate. The filtrate no longer agglutinated a suspension of *C. diphtheriae*. No loss of specific antitoxin from the filtrate was detected following this treatment.

In the second stage of the absorption, non-antitoxic antibodies were flocculated and removed by treatment with excess-iron ultrafiltered culture filtrate as described

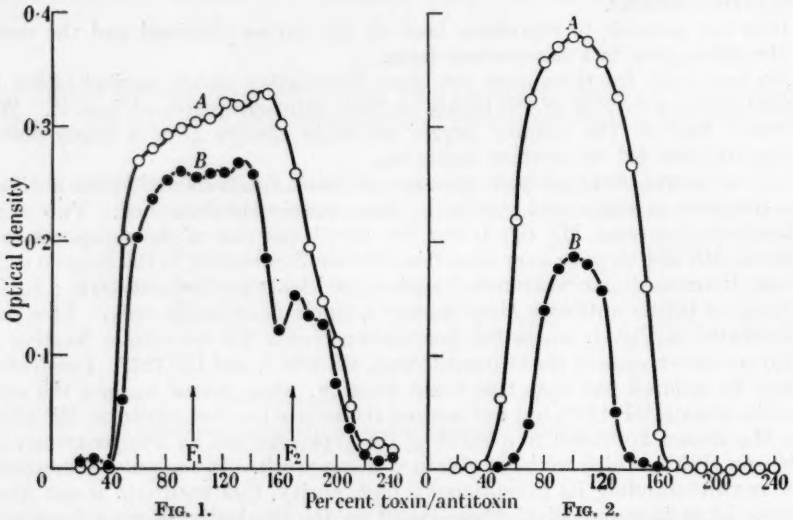

FIG. 1. FIG. 2.

FIG. 1.—The optical density of the floccule precipitates given by toxin fraction MS 460(6).
Curve A for Antitoxin DP 1252 constant at 20 u./ml.
Curve B for Antitoxin MS 453(3) constant at 20 u./ml.

F_1 and F_2 are first flocculating points for the two zones shown by this toxin fraction and the highly purified antitoxin.

FIG. 2.—The optical density of the floccule precipitates given by two absorbed antitoxins MS 381 and P 143.
Curve A: Absorbed ammonium sulphate antitoxin (20 u./ml.).
Curve B: Absorbed peptic antitoxin (20 u./ml.).
Both tested against crude diphtheria toxin.

by Pope and Stevens (1953). In this case optimal flocculation, as determined by trial experiments, was obtained by adding 100 ml. highly concentrated excess-iron culture filtrate to the 1·0 l. filtrate described above. Notwithstanding the production of a bulky floccule precipitate, the loss of specific antitoxin could not be established with certainty, thus confirming the earlier results of Pope *et. al.* (1951) that the excess-iron culture filtrates contain antigens that do not react with the specific antitoxin but do with other antibodies present in antitoxic serum.

For the third stage of the absorption the supernatant, after removal of the excess-iron floccule precipitate, was measured and its antitoxin content determined; to the total number of antitoxin units so found, " peptic-toxin " prepared as

described by Pope and Stevens (1953) was added to give five times this number of Lf units based on the original toxin value of 500 Lf u./ml. This produced in effect a 5-equivalent mixture, i.e., 500 per cent " toxin " to antitoxin units. With this large addition of " peptic-toxin " in one dose no floccule precipitate was produced. It was therefore necessary to separate antigen-antibody complexes and concentrate this material by fractionation with ammonium sulphate.

The " absorbed " antitoxin was therefore diluted until it contained 80 u./ml. and 20 per cent solid ammonium sulphate added. A precipitate was produced and this was filtered off, dissolved and tested for antitoxin ; only a small fraction of the total antitoxin was present in this precipitate. The filtrate, containing the bulk of the antitoxin, was again precipitated by adding more solid ammonium sulphate to a final concentration of 35 per cent and the precipitate filtered off and dialysed free from ammonium sulphate in the usual manner.

Examination of this antitoxin by both *in vitro* and *in vivo* tests showed that the ratio *in vivo*/*in vitro* units was still substantially 1·0, and that there had been no change in this ratio as a result of absorption purification. After purification by absorption in this way the antitoxin (P 143) was examined using the optical method described by Pope and Stevens (1953). The mixtures covering the range 20 to 240 per cent toxin/antitoxin were prepared with the antitoxin constant at 20 u./ml. and the result shown in Fig. 2 (B) was obtained. In this experiment crude toxin, concentrated by ultrafiltration, but not purified in any way, was used. It will be seen that both the ammonium sulphate fraction from this antitoxin (MS 381) when purified by absorption and the peptic-antitoxin (P 143) similarly purified yield symmetrical curves with crude toxin. There is of course a difference in the bulk of floccule precipitate produced ; for the peptic-antitoxin, about a half of the molecule with no antitoxic properties has been split off as a result of pepsin treatment (Pope, 1938 ; Petermann and Pappenheimer, 1941). The figures for the optical density values of the floccules are in the ratio 2 : 1 for the absorbed sulphate fractionated/absorbed pepsin-treated antitoxin (i.e., 0·376/0·187), the antitoxin concentration being the same in each case, namely 20 u./ml. in the mixtures.

After the isolation of absorbed antitoxins, both non-pepsin and pepsin treated, which behaved alike in giving symmetrical flocculation curves with crude toxin and were therefore quite unlike those described by Pappenheimer and Robinson (1937) and Cohen and Pappenheimer (1949) for horse antitoxin, it was obviously necessary to study what has been called the " equivalence zone," where, in a series of mixtures, neither free toxin nor antitoxin is detectable in the supernatants. Pappenheimer and Robinson found that this equivalence zone extended over a wide range of ratios of toxin/antitoxin, and from the fact that the supernatants in this region contained neither free toxin nor antitoxin, assumed that these were quantitatively present in the floccule precipitates. The mixtures which gave curve B in Fig. 2 were therefore examined (*a*) as total mixtures and (*b*) as centrifuged supernatants and the results obtained are shown in Table III. All the results are for tests in guinea-pigs using the intradermal method. It is clear that these toxin/antitoxin mixtures do not show a broad equivalence system as described by Pappenheimer and Robinson. In fact the results show both for total mixtures and for the supernatants a sharply defined end-point, corresponding to the 100 per cent mixture which showed (*a*) first flocculation and (*b*) the peak of the density curve (Fig. 2 B). In the 90 per cent mixture supernatant free

TABLE III.—In Vivo *Results for Total Mixtures and Supernatants with Antitoxin P* 143 *Constant at* 20 *U./ml. and Toxin Varied.*

Per cent toxin/antitoxin.	Toxin or antitoxin detected* in 1·0 ml. of—	
	Total mixture.	Supernatant.
60	2·0 units antitoxin	2·0 units antitoxin
70	1·2 „ „	0·55 unit „
80	*ca.* 0·8 „ „	0·12 „ „
90	0·1 „ „	0·04 „ „
100	Very slightly toxic	Very slightly toxic
110	Toxic *ca.* 5000 MRD/ml.	Toxic *ca.* 5000 MRD/ml.
120	„ >5000 <50,000 MRD/ml.	„ >5000 <50,000 MRD/ml.
130	„ *ca.* 50,000 MRD/ml.	„ *ca.* 50,000 MRD/ml.

* Intradermal tests in guinea-pigs.

antitoxin was detected and at 110 per cent the supernatant was toxic. The 100 per cent mixture supernatant showed only a doubtful slight toxicity. Thus it would appear that for horse antitoxin, from which other non-antitoxic antibodies have been removed by absorption with their corresponding antigens, there is now no broad " equivalence zone " as described by Pappenheimer and Robinson (1937) and Cohn and Pappenheimer (1949). Moreover, the methods employed by these workers to determine the nitrogen associated either with one unit of antitoxin or one Lf unit of toxin, which depended on determination of the nitrogen content of the floccules and the slope of the curve in the " equivalence zone," cannot be applied to antitoxin purified to the extent reported here. There is neither " equivalence zone " nor " straight line portion " of the curve available for these calculations.

The Production of Non-flocculating Antitoxin.

At this point it appeared that we had completed the absorption of all the non-antitoxic antibodies and were now dealing with the flocculation reaction between toxin and antitoxin alone. It was considered desirable to confirm these results and the purification already described was repeated. Unavoidably, the time allowed for reaction (*a*) at the " Fe-antigen " stage before removing the floccules and (*b*) at the " peptic-toxin " stage before fractionating with ammonium sulphate was longer than in the previous experiment. This is a possible factor that needs further investigation. The final product obtained showed a significant difference in properties. Tests showed that the *in vivo* and *in vitro* values were no longer in agreement ; the flocculation value was now considerably lower than the animal value and the ratio Lr/Lf was 1·55. This absorbed antitoxin thus gave two values, one based on flocculation tests and the other on the specific neutralization of toxin in the guinea-pig. A series of mixtures prepared on the basis of 20 *in vitro*

TABLE IV.—In Vivo *Results on Supernatants for Mixtures Based on* 20 in vitro *Units Antitoxin/ml. Antitoxin P* 143/*R.*

Per cent toxin/antitoxin.	Toxin or antitoxin in supernatant.
130	*ca.* 2 units antitoxin/ml.
140	*ca.* 2 „ „ „
150	*ca.* 1 unit „ „
160	Toxic *ca.* 50 MRD/ml.
170	Toxic >50,000 MRD/ml.

u./ml. gave supernatants that showed free antitoxin up to a mixture with the composition 150 per cent toxin/antitoxin, as shown in Table IV.

From these results it is clear that an antitoxin which had originally an *in vivo*/*in vitro* ratio of 1·0 has yielded, after absorption, a product where the *in vitro* test considerably underestimates the specific toxin neutralizing capacity.

Another series of mixtures was prepared based on 20 *in vivo* u./ml., on which both the optical density and the properties of the supernatants were determined.

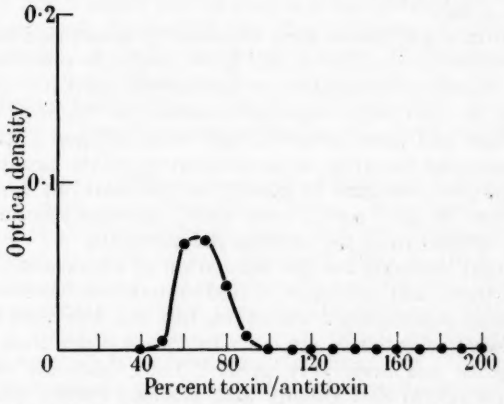

FIG. 3.—The optical density of the floccule precipitates given by antitoxin P 143/R constant at 20 *in vivo* u./ml. tested against crude diphtheria toxin.

The optical density values are shown in Fig. 3. Only a very meagre floccule precipitate was obtained with a peak value at 60 to 70 per cent toxin/antitoxin and no floccule precipitate at 100 per cent. The results of the *in vivo* tests on the supernatants are given in Table V, and these show that at 100 per cent toxin/antitoxin the supernatant was almost free from either toxin or antitoxin; excess antitoxin was present at 90 per cent and excess toxin at 110 per cent toxin/antitoxin. This antitoxin, P 143/R, purified by absorption of non-antitoxic antibodies, again shows the very sharp "equivalence point" observed in the

TABLE V.—In Vivo *Results on Supernatants for Mixtures Based on* 20 In Vivo *Units Antitoxin P* 143/*R per ml.*

Per cent toxin/antitoxin.	Toxin or antitoxin detected in 1·0 ml. of—	
	Total mixture.	Supernatant.
40	Antitoxin 8 u./ml.	Antitoxin 8 u./ml.
50*	,, 6·5 ,,	,, 6 ,,
60*	,, 5 ,,	,, 4·5 ,,
70*	,, 4 ,,	,, 3·3 ,,
80*	,, 2·4 ,,	,, 2·4 ,,
90*	,, 1·2 ,,	,, 1·2 ,,
100	Toxin *ca.* 1250 MRD/ml.	Toxin *ca.* 1250 MRD/ml.
110	*ca.* 25,000 ,,	*ca.* 25,000 ,,
120	*ca.* 125,000 ,,	*ca.* 125,000 ,,

* Floccule precipitate was present only in these mixtures.

previous experiment (Table III) rather than a broad "equivalence zone," but with the important differences that at the *in vivo* "equivalent point" there was no flocculation. Such flocculation as occurred appeared to be related to residual traces of non-antitoxic antibodies and not to the specific toxin-antitoxin reaction.

DISCUSSION.

From the work of Pope and Stevens (1953) and that reported here several points of interest arise :

1. As non-antitoxic antibodies were removed by absorption with their antigens the amount of floccule precipitate in the toxin/antitoxin reaction decreased until finally antitoxin giving no flocculation at the neutral point (*in vivo*) was obtained.

2. The *in vivo/in vitro* ratio remained constant at 1·0 until most of the non-antitoxic antibodies had been removed and then changed ; the *in vitro* value then no longer indicated the true antitoxin content of the product.

3. These antitoxins, obtained by absorption purification, showed an "equivalence point" instead of an "equivalence zone" in their reaction with toxin and in this way they differed from the unabsorbed antitoxin.

The fundamental methods for the estimation of either antitoxin or toxin are based on animal tests, and although of undoubted convenience the flocculation test has not the same significance. Since this test was described by Ramon (1922) several workers have reported *in vivo/in vitro* ratios other than 1·0 and in some cases the divergence has been very great. Thus, Maloney and Weld (1925), Sordelli and Serpa (1925) and Glenny and Wallace (1925) reported differences between animal and flocculation values. Glenny, Pope, Waddington and Wallace (1925) found that the "serum ratio" *in vivo* value/*in vitro* value was often constant for successive samples from the same horse, but usually there was a tendency for the ratio to be higher in early tests and to become constant as immunization proceeded ; unusual serum ratios appeared more frequently among sera of relatively low titre. An antitoxic serum with a very abnormal ratio was reported by Barr and Glenny (1938) ; from this serum they obtained, by ammonium sulphate fractionation, a product with an animal value nearly 20 times greater than its flocculation value. Glenny (1931) states : "The fact that it has been demonstrated without doubt that great discrepancies may sometimes occur between *in vitro* and *in vivo* titrations makes it imperative that confirmatory animal tests should be made upon antitoxin intended for therapeutic use." It is therefore surprising to find that Cohn (1952), criticising the work of Pope *et al.* (1951), should state : "In terms of quantitative immunochemistry the discrepancy between the L+ and Lo, taken together with the large Danysz effect observed after all the antitoxin is combined, signifies that the reaction between diphtheria toxin and horse antitoxin is characterised by a broad "equivalence zone." In further support he quotes Jerne (1951) as having pointed out "that 2 antitoxins could have the same unitage as determined by flocculation reaction and differ markedly when compared by skin neutralization test (*in vivo*) and vice versa." From this it would appear that he considers the results obtained by immunochemical methods to be more fundamental than those obtained by *in vivo* methods. Just how dangerous such an assumption may be is well shown by the results presented in this paper.

The presence of multiple non-antitoxic antibodies in commercial diphtheria

antitoxin, as reported by Pope *et al.* (1951) and further confirmed by Pope and Stevens (1953), was considered to be a major factor in producing the bulk of the Ramon floccule precipitate. An extension of this work now reported shows that it is possible to continue the removal of these non-antitoxic antibodies to a point where an artificial divergence between the *in vivo* and *in vitro* values is produced and there is no longer any flocculation at the true animal neutral point. We previously pointed out the need in this work to avoid large losses of specific antitoxin during purification, and this principle has been adhered to in this work. Admittedly rather larger losses are inevitable when purification is carried to the degree reported here, but the specific loss due to traces of toxin in the antigen systems was no greater; only the mechanical losses due to ammonium sulphate fractionation were involved additionally. The specific loss was of the order of 20 per cent, and the mechanical loss about 10 to 20 per cent. The results reported here are not, therefore, based on the properties of a very small fraction of the original antitoxin.

Marrack (1938) states: "There is considerable evidence that part of the antibody molecules in a given serum are not uniform. When part of the antibody is precipitated by antigen, the antibody molecules left in solution are probably those of lower affinity for antigen and may well differ in their behaviour in the optimum proportions reaction." A possible effect of this kind has been considered but no evidence supporting the view that the absorbed antitoxin is "non-flocculating" because it consists of antitoxin from which special molecules of high avidity or of high valency have been removed has been found. Antitoxin, isolated from mixtures where 20 per cent of the specific antitoxin has been neutralized with crude toxin, does not show this non-flocculating property. Only the most complete removal of non-antitoxic antibodies as described here produces this effect.

In the *in vitro* investigation of the reaction between diphtheria toxin and antitoxin Healey and Pinfield (1935) found a broad range of mixtures with compositions of about 50 to 130 per cent toxin to antitoxin where, after removing the floccules by filtration, no free toxin or antitoxin could be detected in the filtrates by *in vivo* tests. They also found (personal communication) that *in vitro* tests showed the same results. A similar result was obtained by Pappenheimer and Robinson (1937), and we have found (unpublished) that the yield of antitoxin that can be recovered by applying the method of Pope and Healey (1939) to washed floccules from mixtures prepared from non-absorbed antitoxin of composition 60 to 140 per cent toxin/antitoxin is substantially the same. It is therefore clear that with normal or peptic antitoxin the floccules from a broad range of mixtures do contain the toxin-antitoxin complex. The absorbed antitoxins reported here show quite different behaviour, and there may be no flocculation at the neutral point. We have suggested that the toxin-antitoxin complex may be entrained in the gel-precipitate formed by other antigen-antibody systems, and the work reported here further supports this view. In the *in vivo* tests carried out on the absorbed antitoxin no difficulties were experienced in testing the antitoxin value to close limits and there were no indications of any abnormal behaviour.

If it is accepted that antitoxin, freed from other antibodies, has the properties reported in this paper, and does not flocculate with toxin, then the converse should hold, and it should be possible to purify diphtheria toxin to a point where it no longer flocculates with crude antitoxin. Work along this line is in progress.

SUMMARY.

A series of diphtheria toxin fractions of differing purities was used to test the homogeneity of the absorbed antitoxin with a purity of 211,000 units/g. protein previously isolated. It has been shown that this preparation was not homogeneous.

The properties of antitoxin after more thorough removal of non-antitoxic antibodies have been examined. An artificial change in the *in vivo/in vitro* ratio was found, the antitoxin did not flocculate at the *in vivo* " equivalence point," and toxin/antitoxin mixtures showed a sharp " equivalence point " instead of a broad " equivalence zone."

Our thanks are due to Dr. C. L. Oakley for the *in vivo* tests shown in Table III, and to Mr. A. T. Glenny, F.R.S., for helpful discussions.

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THE EFFECT OF OXYGEN LACK ON PENICILLIN-INDUCED PENICILLINASE FORMATION BY *BACILLUS CEREUS*.

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IT has previously been found that cells of a strain of *Bacillus cereus* will form penicillinase at a very greatly increased rate after only transient contact with penicillin (Pollock, 1950). More recently it was shown (Pollock, 1952) that in cultures growing in the log-phase this specific adaptation response to penicillin can be divided into at least three stages. The first stage of " primary interaction " between penicillin and cells is very rapid, being 50 per cent complete in 60 sec., and is thought to entail the formation of some new complex between penicillin and a specific cell receptor, which initiates the adaptation response. There then occurs a " latent phase " of about 15 min. during which relatively little enzyme is formed, followed by the third (" active ") phase when the enzyme is formed at a constant rate determined by the concentration of added penicillin. During the three phases the cells are growing continuously with constant mean generation time, the growth rate being unaffected by the addition of penicillin or by the penicillinase adaptation process in any of its phases. It was found that the temporary substitution of anaerobic for aerobic conditions of culture (by bubbling argon through the culture for 25 min.) caused an immediate cessation of both growth and penicillin production, and as sudden a resumption, without lag, when air was restored. This temporary anaerobiosis had no effect on the primary interaction phase of adaptation, but its action on both the latent and active phases was to cause an immediate " break " in the process, so that the cells, on restoration of air, simply resumed the metabolic processes at the point where they were inhibited when air was first removed.

If the anaerobiosis was prolonged for 100 min., growth re-started on restoration of air, at the same rate as before, without lag ; but penicillinase formation showed a very different effect, which it is the purpose of this report to describe and attempt to interpret.

METHODS.

The strain of *B. cereus* (NRRL 569), medium, inoculation, methods of aerobic and anaerobic incubation were exactly as described in detail previously (Pollock, 1952). Growth was measured turbidimetrically on the Spekker absorptiometer. All experiments were done on 50 ml. lots of logarithmically-growing culture in 1 per cent gelatin-broth, shaking in 250 ml. conical flasks in a 35° water-bath. Penicillin at 1 unit/ml. ($1{\cdot}7 \times 10^{-6}$ M) was added when the culture opacity reached levels corresponding to a cell concentration of between 0·03 and 0·1 mg. dry bacterial wt./ml. When necessary, in order to sharpen the end of the primary interaction phase and allow accurate measurement of the latent phase, penicillinase (final activity 250 µl. CO_2/hr./ml.) was added 60 sec. later in order to destroy all free penicillin within a few seconds. Anaerobiosis was achieved by transferring the culture to a boiling tube and bubbling argon through at 500 ml./min. Penicillinase activity was assayed manometrically (Henry and Housewright, 1947) by measuring the rate of

release of CO_2 from a bicarbonate buffer during the hydrolysis of penicillin to penicilloic acid, 2·5 ml. samples being removed from the culture at 5 or 10-min. intervals and further enzyme formation stopped by addition of 0·0008 M oxine (*see* Pollock, 1950). Ordinary penicillin used was the sodium salt of benzyl penicillin (Glaxo Ltd.) having an activity of 1670 units/mg. Radioactive ^{35}S-labelled benzyl penicillin of specific activity about 0·18 μc./unit was prepared by C. J. Perret (Pollock and Perret, 1953).

RESULTS.

"Rebound" and "Reversion."

When anaerobic conditions were applied to a log-phase culture of *B. cereus* 60 min. after addition of penicillin, and allowed to continue for 100 min., the rate of penicillinase formation following restoration of air was temporarily increased (nearly 4-fold) before it settled back to the previous rate shown by a control aerobic culture and the original culture before anaerobiosis was started (see Fig. 1*d*). A similar "rebound" effect is shown in Fig. 1*c* when anaerobiosis was begun 40 min. after penicillin. When, however, the 100 min. anaerobiosis was begun only 20 min. after the penicillin, a second phenomenon occurred (Fig. 1*a* and 1*b*). The "rebound" effect was still marked, but the original rate of penicillinase production was never attained. There had been a loss or "reversion" of the ability to form penicillinase, to the extent of about 65 per cent (*i.e.*, the new rate was only 35 per cent of the original rate), and this was apparently never regained. This reversion effect was even more marked when the anaerobiosis was begun only 2 min. after addition of penicillin (i.e., at the beginning of the "latent phase"—see Fig. 2, curve C′). Reversion here has occurred to the extent of at least 85 per cent compared to the control "aerobic" curve (A). The "rebound" phenomenon, however, was absent. The anaerobiosis in this experiment lasted for 120 min., but it was subsequently shown that there was no significant difference between this and 100 min. as far as penicillinase formation was concerned.

Fig. 2 also shows the relatively insignificant effects of only 12 or 25 min. anaerobiosis (curves A′ and B′ respectively); and also the similar, but less pronounced reversion (together, of course, with the "rebound" phenomenon) occurring when anaerobiosis was begun 20 min. after the penicillin (C″) in the same experiment, with an aliquot from the same culture as that used for C′. The progressive effect of prolonging the anaerobic conditions (in this case begun 20 min. after penicillin) is best shown in Fig. 3, illustrating three separate experiments, where reversion after 50 min. (B′) was only about 50 per cent and, after 20 min. (A′), undetectable. All three curves A′, B′ and C′ show some "rebound," although it is more marked in C′, and barely significant in A′.

It should here be explained that in the experiment illustrated by Fig. 1*d* the penicillin has to be added at the low cell density of 0·03 mg./ml. (compared to 0·1 mg./ml. as in the experiment shown in Fig. 1*b*), in order to allow the penicillinase samples in B to be taken within the log-phase, beyond which linear production of enzyme does not persist. It might thus be argued that the extent of reversion is really a function of the cell concentration at the point when penicillin is added—particularly as this latter will also determine the rate of penicillinase production for a given concentration of added penicillin. This, however, is not the case. Fig. 1*a* shows that exactly the same extent of reversion occurred

when penicillin was added at a cell concentration of 0·03 mg./ml. as at a concentration of 0·1 mg./ml. (Fig. 1*b*), although of course in the latter the slope of the linear phase in the control aerobic culture (A) was over three times that of the former.

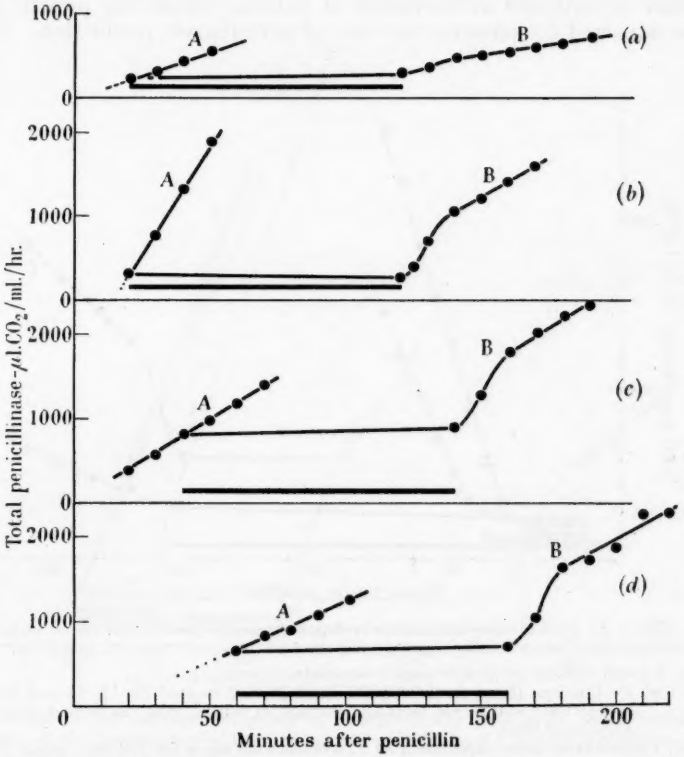

FIG. 1.—Effect on penicillinase formation by a logarithmically growing *B. cereus* culture of 100 min. anaerobiosis begun at different times after addition of 1 unit/ml. penicillin.

A. Control cultures.
B. Cultures treated for 100 min. with argon bubbling through at 500 ml./min. (indicated by broad horizontal line).

(*a*) Argon begun 20 min. after penicillin (at cell conc. 0·03 mg./ml.).
(*b*) ,, ,, 20 ,, ,, ,, (,, ,, 0·1 ,,).
(*c*) ,, ,, 40 ,, ,, ,, (,, ,, 0·06 ,,)
(*d*) ,, ,, 60 ,, ,, ,, (,, ,, 0·03 ,,)

It thus seems possible to conclude that (*a*) no reversion occurs during anaerobiosis if the cells have been growing aerobically for 40 min. or more after addition of penicillin, and (*b*) rebound is only shown by cells which have passed through the latent phase and have begun active enzyme production.

Specificity.

It is obvious at this stage that these effects would be of little immediate interest if they were not specific; if, for instance, similar variations occurred in cell growth or general metabolism following anaerobiosis. Any variations in the "fundamental" metabolic processes of the cell such as energy supply or assimilation of nutrients or formation of building blocks for protein synthesis would be expected to influence the rate of penicillinase production. However,

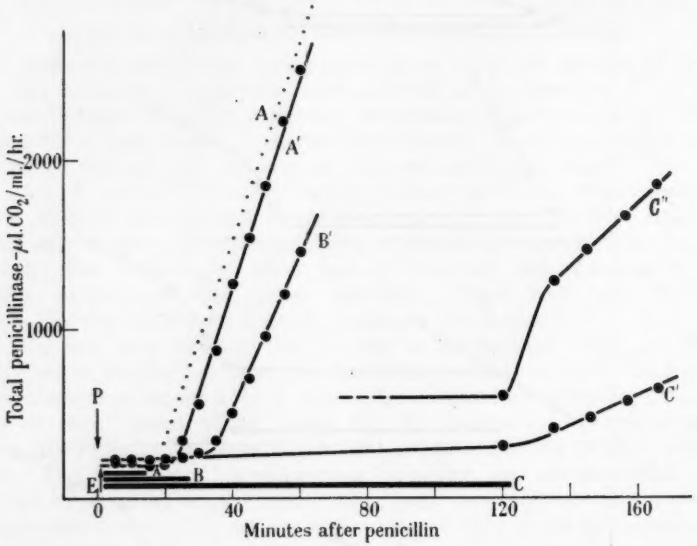

FIG. 2.—Effect on penicillinase formation by logarithmically growing *B. cereus* cultures of different periods of anaerobiosis begun 2 min. after addition of 1 unit/ml. penicillin.

A. Control culture (no anaerobiosis)—separate experiment.
A', B', C'. Cultures (from three separate experiments) treated for 12, 25 and 120 min. respectively with argon bubbling through at 500 ml./min., as indicated by broad horizontal lines.
C''. Culture from same experiment as C', treated with argon for 100 min. begun 20 min. after addition of penicillin, for comparison with C', when argon was begun 2 min. after penicillin.
P. Addition of penicillin.
E. Addition of penicillinase 250 μl./ml./hr. to destroy free penicillin.

in this instance the effect does appear to be specific. As mentioned before and shown in Fig. 4, its effect on growth is simply to cause a "break," the cells growing logarithmically as before, without lag or "rebound," at the previous rate as soon as air is restored. This effect on growth is quite independent of the addition of 1 unit/ml. penicillin. Neither was there any effect on O_2 consumption (Fig. 5). In this experiment 3·0 ml. samples were removed from the aerobically shaking culture (A) at the same moment as anaerobiosis was applied to B, put in a Warburg manometer cup and O_2 uptake measurements begun, after 4 min. preliminary equilibration, as rapidly as possible, the whole process up to the first recorded reading taking 7 min. A similar sample (B) was studied

after the end of 100 min. anaerobiosis. The turbidity of the culture in the Warburg cups after 48 min. was compared with that of the bulk culture left shaking in the 250 ml. conical flask (as in all adaptation experiments) and found to be identical. It can therefore be reasonably concluded that the rate of O_2 uptake in this experiment was the same as that occurring in the bulk cultures just before and just after 100 min. anaerobiosis. It can be seen that the curves are almost

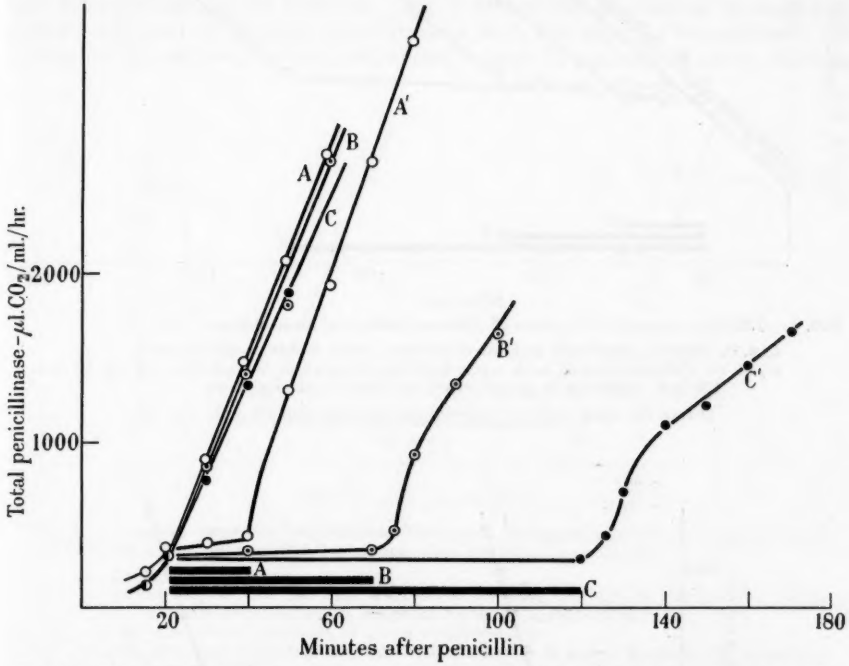

FIG. 3.—Effect on penicillinase formation by logarithmically growing *B. cereus* cultures of different periods of anaerobiosis begun 20 min. after addition of 1 unit/ml. penicillin.

A, B, C. Control cultures (three separate experiments).
A′, B′, C′. Cultures treated with argon bubbling through at 500 ml./min. for 20, 50 and 100 min. respectively as indicated by broad horizontal lines.

identical. Finally, the ability of the cells to respond to penicillin is quite unaltered after 100 min. anaerobiosis. Fig. 6 shows that the curve of penicillinase production (viz., the length of latent phase and slope of linear phase) when penicillin is added immediately after 100 min. of argon (B) is almost exactly the same as that of the control aerobic culture (A) after penicillin addition, both cultures of course having the same opacity.

Effect of Suppression of Metabolism by Cold.

If cell metabolism was inhibited by rapid cooling of the culture to 0° and maintenance at that temperature for 100 min., growth recommenced on trans-

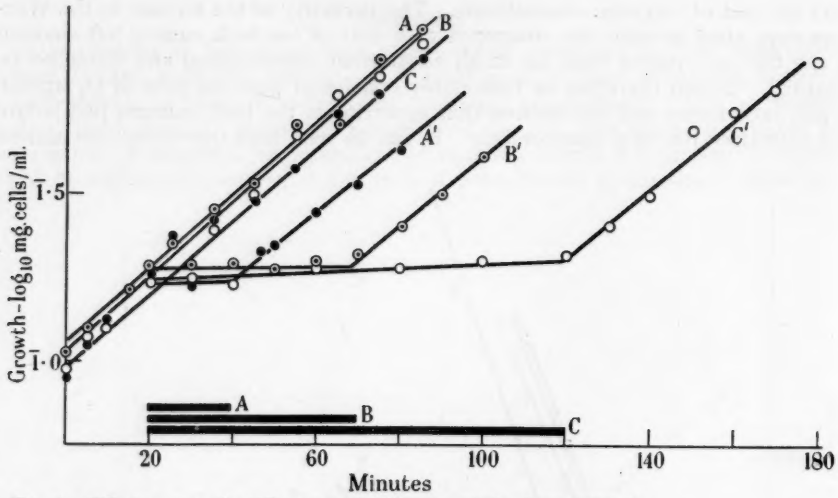

FIG. 4.—Effect on growth of *B. cereus* of different periods of anaerobiosis.

A, B, C. Control aerobically incubated cultures (three separate experiments).
A′, B′, C′. Cultures treated with argon bubbling through at 500 ml./min. for 20, 50 and 100 min. respectively as indicated by broad horizontal lines.

(From the same series of experiments as those illustrated in Fig. 3.)

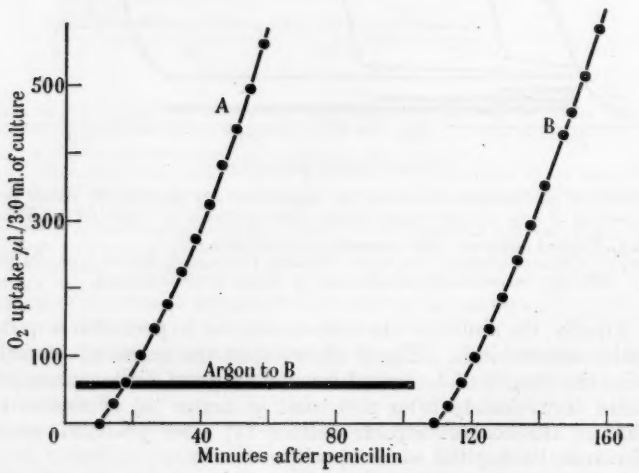

FIG. 5.—O_2 uptake of logarithmically growing *B. cereus* cultures before and after 100 min. anaerobiosis begun 2 min. after addition of 1 unit/ml. penicillin.

A. Control.
B. Culture treated for 100 min. with argon bubbling through at 500 ml./min., as indicated by broad horizontal line.

ference to 35° at the original rate as with restoration of air after 100 min. anaerobiosis. The only difference was that there was a slight lag on transference to 35° before a constant rate was attained, possibly due to the difficulty of instantaneously raising the temperature of the whole culture to 35° from 0° despite rapid agitation in pre-warmed vessels. But there was no reversion or rebound effect on penicillinase production. Formation of the enzyme re-started, after only a short lag, at the same rate as before cooling, almost as if nothing at all had occurred during the interval. Fig. 7 shows the absence of reversion in a culture inhibited by cooling in comparison with the effect of anaerobiosis, after which the rate of penicillinase production is only 20 per cent as great—although

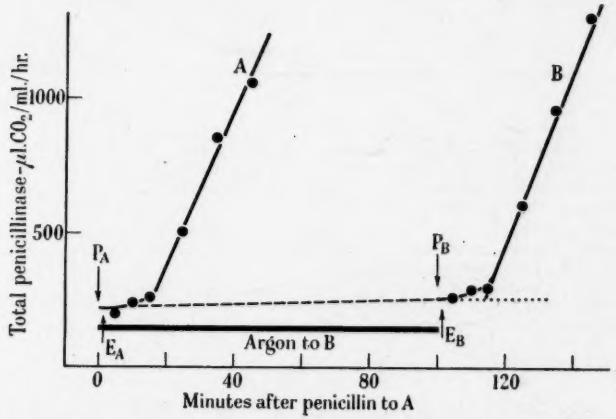

FIG. 6.—Adaptive response (penicillinase formation) of logarithmically growing *B. cereus* cultures to 1 unit/ml. penicillin before and after 100 min. anaerobiosis.

A. Control.
B. Culture treated for 100 min. with argon bubbling through at 500 ml./min. before addition of penicillin, as indicated by broad horizontal line.
P_A, P_B. Addition of penicillin to A and B respectively.
E_A, E_B. Addition of penicillinase 250 μl./ml./hr. to A and B respectively to destroy free penicillin.

rates of growth were identical. Fig. 8 shows the absence of rebound following 100 min. at 0° begun 60 min. after penicillin, and should be compared with Fig. 1*d*, which shows the effect of 100 min. anaerobiosis, also begun 60 min. after penicillin.

Retention of Penicillin S on Cells.

The specific fixation of penicillin sulphur on cells is known to be associated with the inducing effect of penicillin on penicillinase adaptation (Pollock and Perret, 1951). It was therefore thought possible—though unlikely—that the reversion effect might be due to the loss of bound penicillin, or some S-containing inducer substance, from the cells during anaerobic incubation. A simple diffusion of penicillin or penicillin-like substances out of the cells had already been shown to be improbable, since this would be expected to occur to some extent during 100 min. treatment at 0° (conditions under which no reversion occurs). In

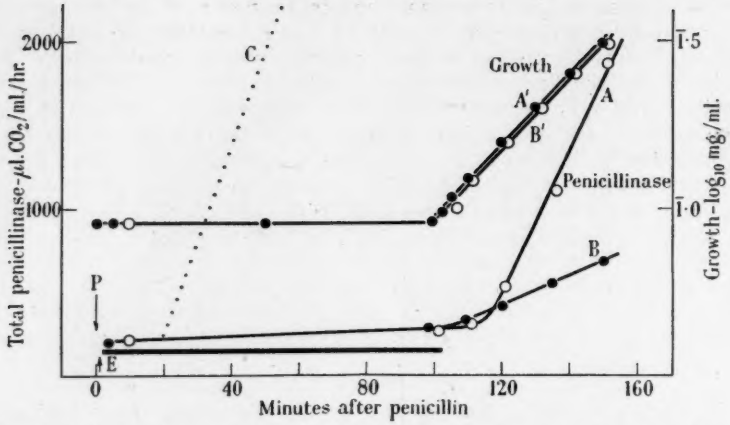

FIG. 7.—Comparison of growth (A′, B′) and penicillinase formation (A, B) of two logarithmically growing *B. cereus* cultures following 100 min. treatment, begun 2 min. after addition of 1 unit/ml. penicillin, at 0° and anaerobically at 35° respectively.

A, A′. After 100 min. at 0°.
B, B′. After 100 min. treatment with argon bubbling through at 500 ml./min. at 35° (as indicated by broad horizontal line).
C. Control untreated culture (separate experiment).
P. Addition of penicillin (to both cultures).
E. Addition of 250 μl./ml./hr. penicillinase (to both cultures) to destroy free penicillin.

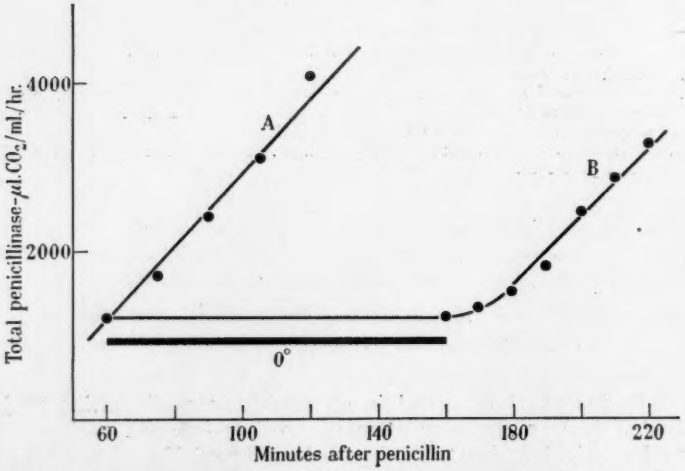

FIG. 8.—Effect on penicillinase formation by a logarithmically growing *B. cereus* culture of 100 min. treatment at 0° begun 60 min. after addition of 1 unit/ml. penicillin.

A. Control.
B. Culture treated at 0° for 100 min. as indicated by broad horizontal line.

order to be certain that reversion could not be explained simply by loss of penicillin previously bound to the cells, a 300 ml. log-phase culture was treated with 0·1 unit/ml. ^{35}S-labelled penicillin, and penicillinase was added 60 sec. later to convert all free penicillin to penicilloic acid. At this concentration, cells fix about eight times more S from penicillin than from penicilloic acid. The culture was then divided into three lots and treated as follows:

(*a*) Immediately centrifuged and the cells washed three times with water.

(*b*) Incubated aerobically for 40 min., then anaerobically for 100 min. at 35°, followed by centrifugation and washing of cells.

(*c*) Incubated anaerobically for 100 min., then aerobically for 40 min., followed by centrifugation and washing of cells.

All cells were thus in contact with penicillin for 60 sec. and with penicilloic acid (formed from the penicillin by enzymic hydrolysis) for the rest of the period of incubation, during which they would be growing and the total S fixed (by uptake from the penicilloic acid) would inevitably increase. A similar experiment was therefore done as a control using the same concentration of penicilloic acid (prepared by mixing the penicillinase with ^{35}S-labelled penicillin and allowing 5 min. incubation at 35° before addition to the cells). The six lots of cells were freeze-dried on discs and fixed S determined by measurement of radioactivity (*see* Pollock and Perret, 1951). It is known that no reversion effect occurs in (*b*) and 85 per cent reversion in (*c*), so that if the effect were due to loss of penicillin-S, there should be a big difference in S fixed by cells from (*b*) and (*c*).

In the Table the significant figures are in the last column, which shows the amount of S retained by cells specifically from penicillin expressed in terms of

TABLE.—*Retention of Penicillin-S by Penicillin-treated Cells of* B. cereus *during Subsequent Aerobic Growth and Anaerobic Incubation at* 35°.

Cells.	Subsequent treatment after addition of ^{35}S-labelled penicillin or penicilloic acid.	Treatment of cells.			10^{10} atoms S specifically retained from penicillin per mg. original cells.
		0·1 unit/ml. penicillin for 60 sec.		0·1 unit-equiv./ml. penicilloic acid.	
		10^{10} atoms S fixed per mg. cells.	Cell conc. after incubation (mg./ml.).	10^{10} atoms S fixed per mg. cells.	
(a)	*nil*	23·7 (±1·61)	0·20	3·4 (±0·97)	20·3 (±1·88)
(b)	40 min. aerobic growth followed by 100 min. anaerobic incubation	13·0 (±1·06)	0·35	2·9 (±0·62)	19·0 (±2·13)
(c)	100 min. anaerobic incubation followed by 40 min. aerobic growth	14·4 (±1·10)	0·35	3·4 (±0·58)	18·8 (±2·12)

For details see text. Figures in brackets = S.D.

the original amount of cells present at the time the penicillin was added. These figures have been obtained by subtracting the penicilloic acid-S fixed per mg. cells from the total S fixed per mg. cells after treatment with penicillin for 60 sec. followed by enzyme, and multiplying by the ratio of cell concentration after incubation to original cell concentration.

It is clear that there was no significant difference between the total amounts of penicillin-S fixed on cells of the three cultures. Thus, not only was penicillin-S

retained to the same extent whether aerobic incubation preceded anaerobic incubation following addition of penicillin, or *vice versa*, but there was, in fact, no significant loss of penicillin S during the total 140 min. incubation and metabolism after addition of penicillin and its removal from the medium by enzymic hydrolysis.

DISCUSSION.

There are apparently two separate phenomena taking place during the 100 min. anaerobic incubation period. The first is the progressive loss of the potential ability to form penicillinase rapidly which had been acquired during the 60 sec. contact with penicillin. This has been referred to as " reversion." This reversion does not, however, take place if the cells have been allowed at least 40 min. aerobic metabolism after addition of penicillin, and is apparently lost less quickly the longer the cells have been able to metabolize aerobically following the penicillin. In other words the new property of " penicillinase-competence " which is known to develop during the latent phase does not reach complete metabolic stability for about 40 min. The second phenomenon is the process underlying the transitory rapid production of penicillinase immediately after restoration of air referred to as " rebound," and lasting approximately 15–20 min. This effect is not shown unless the cells have passed through the latent phase and reached the phase where active enzyme is produced rapidly. Analogous changes are not shown by growth, O_2 uptake or penicillinase adaptability of the cells, and it is fair to conclude that the processes underlying them are specific for penicillinase formation. Moreover, the effect of anaerobiosis is not that of a simple metabolic pause, such as occurs presumably during the 100 min. treatment at 0°, and which does not result in any permanent alteration in rate of penicillinase production. Nor is it associated with any significant loss of penicillin-S previously bound to the cells during the initial phase of primary interaction.

The effect is thus due to a metabolic interference with the specific mechanism of adaptation, causing (*a*) the progressive loss of one of the factors essential to the stages of adaptation following primary interaction before the new property has become stabilized, and (*b*) transient increase in rate of active enzyme formation. It is useless at this stage to speculate further on the mechanism underlying reversion. It illustrates only too obviously the complexity of the adaptation mechanism. In order to obtain a culture capable of maximal penicillinase formation in the absence of free penicillin (fully " penicillinase-competent "), it is apparently necessary not only to pass through the phase of primary interaction and the latent phase, but to allow aerobic metabolism for another 25 min. or so in order to " stabilize " the newly acquired property. It is of interest that these phenomena of stabilization and reversion are, in some ways, analogous to those reported by Pryce and Hinshelwood (1947), who showed that the stability of the proflavine-resistance acquired by *Bacterium lactis aerogenes* increased with the number of previous subcultures in the presence of proflavine ; although of course such genetical changes occur very slowly over a period covering several subcultures, and not in the course of a few minutes (involving at the most about 1 cell generation time) as with penicillinase adaptation in *B. cereus*. Further work may show the two types of effect to be more closely related than would at first appear likely.

Comment on the "rebound" effect can be somewhat less vague. Within the limits of these experiments it has been shown to be specific, and thus is unlikely to be due to changes in general cell metabolism, or to assimilation of nutrients from the medium or to accumulation of some non-specific precursor, for in that case some effect on growth or O_2 uptake would be expected. It might, however, still be argued that if some non-specific precursor were not the rate-limiting factor for either growth or O_2 uptake, its temporary accumulation would not necessarily cause an acceleration of these processes, although it might still stimulate others. The extent of the specificity of some hypothetical precursor can thus ultimately only be gauged by how far it can be shown that the effects of its accumulation are restricted to specific metabolic functions.

In so far as the rebound is a specific effect, it could therefore be caused either by an increase in the catalytic activity of the specific substance or mechanism which determines the rate of enzyme production during the linear phase, or by the formation of some specific enzyme precursor directly in the chain of enzyme biosynthesis. Although it is impossible to decide definitely between these two alternatives the latter appears the more likely, since it would be on just such a hypothesis that a *transient* increase in enzyme production rate would be expected. If, for instance, formation of a direct enzyme precursor were only partially inhibited by O_2 lack, while the formation of active enzyme were completely prevented, precursor would accumulate during anaerobic incubation, and on restoration of air there would be an immediate increase in velocity of the reaction: precursor $\rightarrow$ enzyme (above that existing before anaerobiosis had begun). Thus, a steady production of enzyme (at the former rate) would not be reached until the excess precursor had been converted into enzyme and the steady state concentration regained. On the hypothesis of stimulation of the catalytic mechanism it would be necessary to explain both how it came to be stimulated by the preceding anaerobiosis in the first place, and why the original activity was restored after 20 min. aerobic metabolism.

The possibility that the formation of a specific enzyme precursor, produced as a result of the intervention of the inducer (penicillin) in cell metabolism, might be the explanation of the latent phase itself has already been considered (Pollock, 1952). If true, and if, indeed, formation of a precursor is not completely inhibited during anaerobiosis, the length of the latent phase following the 100 min. treatment with argon begun immediately after induction by penicillin would be expected to be shortened. This does in fact appear to be the case, since in two separate experiments the duration of the latent phase after 100 min. anaerobiosis was 6 and 9 min. respectively, though it must be admitted that the accuracy of such measurements, with a low rate of active enzyme formation, is inevitably not very great. No such shortening of the lag—nor indeed any change in the rate of penicillinase formation—occurs if the 100 min. anaerobiosis takes place before the addition of penicillin (Fig. 6). If, therefore, a specific enzyme precursor does in fact accumulate during anaerobiosis, it must be of a type whose production depends upon the addition of penicillin. It is thus clearly differentiated from the type of specific enzyme precursor illustrated by the "Pz" protein of *Bact. coli* discovered by Cohn and Torriani (1951), which, though closely related to β-galactosidase immunologically, is present in unadapted cells before addition of the inducer. Thus the idea of the formation of some specific enzyme precursor is not inconsistent with the evidence available. Its simplicity and its ability to account for three distinct

phenomena: the latent phase, the "rebound" and the shortening of the latent phase after anaerobic metabolism, make it a reasonable working hypothesis to serve as a basis for further investigation.

Superficially the picture given by these results appears complicated. Although it is easy to speculate on the possible biochemical mechanism underlying this adaptation process, it is obviously too early to do so with any degree of assurance. The value of this work lies rather in the demonstration of the specificity of the variations that occur and the recognition, which it allows, of the possibility of dividing the process into different stages which can be separately investigated and characterized.

SUMMARY.

If a log-phase culture of *B. cereus* is incubated for up to 25 min. anaerobically, cell growth and penicillinase formation previously induced by addition of penicillin are immediately interrupted, but begin again, without lag, at the same rate as previously when air is restored.

When the period of anaerobiosis is prolonged for 100 min. the rate of penicillinase formation on restoration of air is found to have been profoundly influenced by specific metabolic processes which have occurred during anaerobic incubation. No such changes occur in growth rate, O_2 uptake or adaptability to further addition of penicillin.

The specific processes occurring during anaerobiosis result in two apparently distinct phenomena:

(*a*) A lowering of the rate of penicillinase formation ("reversion") during subsequent aerobic incubation, the extent of which increases with the duration of anaerobiosis, and decreases with the interval between addition of penicillin and the beginning of anaerobiosis. If aerobic incubation is allowed for at least 40 min. after addition of penicillin, no reversion occurs.

(*b*) A temporarily increased rate of penicillinase formation following restoration of air ("rebound") lasting 15-20 min., before the new linear phase is attained. Unless the culture has reached the linear phase of active enzyme production before anaerobic incubation is begun, no "rebound" subsequently occurs. It is suggested that this effect might possibly be due to the accumulation during anaerobiosis of a specific enzyme precursor whose synthesis is only partially inhibited by O_2 lack.

I wish to express my gratitude to Miss Daphne Towler for patient and expert technical assistance.

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THE INTERACTION OF ANTIGENS AND ANTIBODIES IN THE PRESENCE OF LOW CONCENTRATIONS OF SALT.

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IT is commonly accepted that mixtures of antigen and antibody do not flocculate when the salt concentration is below a certain somewhat indefinite minimum. Little work appears to have been done on the matter. Bordet (1899) sensitised bacteria with antiserum and washed the agglutinated bacteria with distilled water; he found that the sensitised bacteria were not re-agglutinated if re-suspended in distilled water and were re-agglutinated if suspended in saline. Marrack and Smith (1930) found that the minimum concentration of NaCl needed for re-flocculation of diphtheria toxin-antitoxin floccules, washed and re-suspended in distilled water, was 0·03 N. Eagle (1930) did somewhat similar experiments and found that 0·02 N NaCl was needed for complete re-agglutination of *Salm. typhi*, and 0·01 N NaCl for re-agglutination of sensitized red blood cells and for re-flocculation of preformed antigen-antibody precipitates in which the antigen was horse serum. We have repeated this type of experiment with the precipitate formed by ovalbumin and antiserum. The precipitate was washed with saline and then with distilled water; it was suspended in distilled water and dialysed in a cellophane sac against repeated changes of distilled water for 4 days. The sac was repeatedly inverted in order to re-suspend the precipitate which repeatedly settled. The suspension was then removed from the sac and centrifuged; no protein was detected in the supernatant fluid with sulphosalicylic acid. In this instance, therefore, the precipitate was not re-dispersed when the salt concentration was low.

The amount of salt needed to produce flocculation or agglutination in mixtures of antigen and antibody may be very different from the amounts needed to re-flocculate or re-agglutinate re-suspended floccules or agglutinates that have formed in the presence of salt.

The only experiments in which direct flocculation or agglutination in mixtures of antibody and antigen were studied, rather than re-flocculation or re-agglutination of preformed floccules or agglutinates, were until recently those of Schmidt (1930*a*) and Duncan (1937). Schmidt used purified diphtheria toxin which contained little salt. Toxin (1 ml.) diluted with saline (9 ml.) mixed with undialysed antitoxic serum (0·045 ml.) flocculated in 35 min.; toxin (1 ml.) diluted with distilled water (9 ml.) mixed with undialysed antitoxin flocculated in 90 min.; so that flocculation occurred in salt concn. below 0·01 N. Mixtures of toxin and antitoxin, dialysed free from salt, did not flocculate in 24 hr. Duncan (1937) found that the amount of anti-H serum needed for complete agglutination of *Salm. typhi* increased as the NaCl concn. fell from 0·02 to 0·0011 N; in the

O-agglutination of *Shig. dysenteriae* the prozone increased as the NaCl concn. fell from 0·07 to 0·004 N ; with 0·002 N NaCl the bacteria were not agglutinated.

If the failure to flocculate or agglutinate in low salt concn. is due to repulsion between oppositely charged antigen and antibody molecules or between antigen-antibody complexes, the amount of salt needed to produce flocculation or agglutination should depend on the charges on antigen and antibody and therefore on the pH of the mixtures. When protein solutions are dialysed against distilled water the pH of the solution approaches the isoelectric point of the protein and the overall charge on the protein falls. The pH was not measured in any of the experiments quoted.

According to Bordet's theory of agglutination and precipitation, antigen and antibody combine even in very low salt concn. (1st stage), but a minimal concn. of salt is needed for the agglutination or precipitation of the antigen-antibody compounds formed (2nd stage). However, Coulter (1920–21) and Duncan (1937) found that the amounts of antibodies that combined with particulate antigens were much reduced when the concn. of salt was lowered. Schmidt (1930*b*) inferred that diphtheria toxin and antitoxin did not combine when the salt concn. was low, as he found that they did not show the Danysz phenomenon in low salt concn. However, the Danysz phenomenon may be attributed to aggregation of the antigen-antibody compounds into larger complexes, which may be too small to settle. It cannot be inferred that toxin and antitoxin have not combined even when the Danysz phenomenon has not been observed.

The only direct method suitable for detecting combinations of antigen and antibody in low salt concn. is observation of the change of light-scattering by a solution of antibody on addition of antigen. We have therefore studied the relation of precipitation in mixtures of antigen and antibody to salt concn., with attention to the pH, and the change in light-scattering on adding antigen to a solution of antibody in low salt concn.

MATERIALS AND METHODS.

Solutions of antibody and antigen.

The antigens used were Armour's crystalline bovine serum albumin (B.S.A.), crystalline horse serum albumin (H.S.A.) and crystalline ovalbumin (Ov.). Antisera were made by immunizing rabbits with intramuscular injections of the antigens adsorbed on aluminium hydroxide. Gamma globulin fractions were prepared from the antisera by Kekwick's (1940) method, slightly modified (Marrack, Hoch and Johns, 1951). Buffer solutions were made with mixtures of 0·067 M Na_2HPO_4 and KH_2PO_4 with distilled water, re-distilled in glass, boiled to remove CO_2 and cooled. The antigens and γ-globulin solutions were dialysed for 5 days at 4° against dilute buffer solutions (used in order to prevent the pH from falling) or against re-distilled water, boiled to remove CO_2 and cooled. The external fluids were changed repeatedly. When the external fluid was a buffer solution a very slight ppt. formed during dialysis ; this was removed by centrifuging. When the external fluid was distilled water a considerable gelatinous deposit settled at the bottom of the dialysing sac. After dialysis the contents of the sac were brought to the pH desired by addition of 0·01 N NaOH ; most of the gelatinous deposit then dissolved ; any remaining undissolved was removed by centrifuging. The dialysed γ-globulin and antigen solutions were further diluted with buffer solutions or boiled distilled water. The Na concn. given in the table were measured by Dr. J. R. K. Preedy by flame photometry.

All receptacles used were Pyrex, Hysil or Monax glass and were cleaned with chromic acid, washed out well, filled with distilled water and left to soak overnight before emptying and drying. When mixtures were to be kept for several days precautions were taken to avoid contamination with bacteria.

Effect of varied salt concentrations on precipitation.

When the effect of varying concn. of salt was studied small volumes of 0·1 N NaCl were discharged into small tubes from an Agla syringe, 0·5 ml. of dialysed antibody solution was added so that it mixed with the NaCl solution; the antigen was then added. The mixtures were left at room temp. for a few hr. and then kept at 4°.

Precipitation curves.

Varying amounts of antigen were added to constant amounts of antibody solution. The mixtures were kept at room temp. for a few hr. and then for at least 3 days at 4°. They were then centrifuged, the ppt. were washed twice with cold 0·9 per cent NaCl solution and the protein in the ppt. measured by the method of Heidelberger and MacPherson (1943*a*, *b*).

Light-scattering.

The general arrangement of the apparatus used is shown in Fig. 1. It consists basically of a light-tight box divided into two compartments. One side houses the photomultiplier tube P and the other contains the cell holder, lens, filter and selenium cell. The light source, which is a mercury arc lamp, is carried in an external housing.

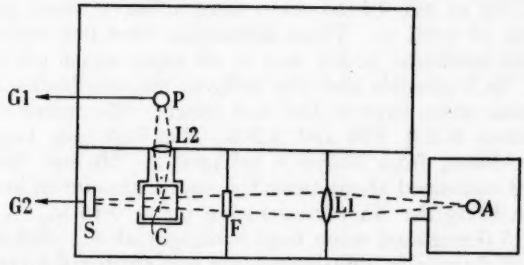

FIG. 1.—Light-scattering apparatus.

A. Mercury arc lamp (Phillips 80 w.).
C. Cell.
F. Filter, Wratten No. 77.
G1, G2. External galvanometers.
L1, L2. Lenses.
P. Photomultiplier tube, 931A.
S. Selenium photocell.

Light from the arc lamp A is focused by the lens L1 to give a slightly converging beam. This passes through a Wratten No. 77 filter which selects the 5460 Å line (plus about 0·5 per cent of the yellow line). The monochromatic beam traverses the central portion of the cell and falls on the selenium cell S. This is used for monitoring the primary beam. Light scattered at 90° is collected by the lens L2 and strikes the photomultiplier P. The anode current of the photomultiplier is measured in the galvanometer G1, which is provided with a backing off circuit and a series of switched shunts by which the sensitivity can be varied over a range of $1:10^{-4}$. The output from the selenium cell is measured in galvanometer G2.

In order to calibrate the instrument in terms of reduced intensity, pure carbon disulphide was used as a working standard. This substance has a reduced intensity of $4{\cdot}4 \times 10^{-5}$ (Campbell, Blaker and Pardee, 1948).

For solutions of proteins, that have the same concn. by wt., the amount of light scattered at an angle of 90° to the incident beam is approximately proportional to the size of the molecules:

$$R_{90} = KcM^*$$

where R_{90} = reduced intensity = $\frac{I_{90}}{I_0} r^2$.

I_{90} = the intensity of light scattered at angle of 90° to the incident beam at distance r.
I_0 = intensity of light transmitted in the direction of the incident beam.
K = constant.
c = concn. of the protein by wt.

The mol. wt. of bovine serum albumin (Armour, crystalline), crystalline pepsin and bovine γ-globulin (Armour) found by this method were 65,000, 33,000 and 180–200,000, respectively.

RESULTS.

The concn. of Na needed to cause flocculation in mixtures of antigen and antibody in equiv. amounts varied with different preparations (Table). Thus, with anti-B.S.A. (P2), a ppt. formed in one day, at pH 6·95, with a Na concn. of only 0·0003 N; and with B.S.A. (P34) at pH 7·0, in an experiment not shown in the Table, as much ppt. formed with a Na concn. of 0·0026 N as with a concn. of 0·15 N. On the other hand, with anti-H.S.A. (P55), at pH 6·9, and Na concn. 0·002 N, no ppt. formed in 3 days and the solution became only slightly cloudy. With anti-Ov (P26) at pH 7·1 no more than a heavy cloud formed in 4 days with a Na concn. of 0·001 N. These differences were not due to differences in the concn. of the antibody, which was in all cases about 0·8 mg. of antibody protein per ml. It is possible that the antigens and antibodies may be changed to varying degrees when kept in low salt concn. We found evidence of such changes in solutions B.S.A. P34 and B.S.A. that had been kept in presence of 0·0026 N kation concn. from March 4 to April 2. No ppt. formed in 6 days in a mixture that contained about twice the equiv. amount of antigen and added NaCl solution to bring the Na concentration up to 0·15 N. A similar mixture made on March 5 flocculated when kept overnight at 4°. Schmidt (1930*a*) also found evidence of changes in diphtheria toxin and antitoxin after dialysis against a low salt concn.

In all the experiments a ppt. formed in 3 days or less when the Na concn. was over 0·003 N, even when the pH was as high as 7·6, if antigen was not in excess.

Effect of hydrogen ion concentration.

Change of pH from 6·8–6·9 to 7·6 had a considerable effect. Thus, with Na concn. 0·0026 N, an equiv. amount of B.S.A. added to 0·5 ml. of anti-B.S.A. (P34) formed as much ppt. in 3 days at pH 6·8 as was formed by the same amounts of antigen and antibody in presence of 0·15 N NaCl. But at pH 7·6 the mixture in 0·0026 N Na was quite clear after 2 days and very slightly cloudy after 20 days; on adding 15 per cent NaCl solution to bring the concn. up to 0·15 N as much ppt. formed as was formed when antigen and antibody were mixed in presence of 0·15 N NaCl.

In the Table anti-B.S.A. (P2), with an equivalent amount of B.S.A., flocculated in 2 hr. at pH 6·95 with Na concn. 0·00063 and formed a ppt. in 1 day; at pH 7·6 a similar antigen-antibody mixture in the same Na concn. was slightly

* A more exact equation is

$$\frac{Kc}{R_{90}} = \frac{1}{M} + 2Bc,$$

where B is an interaction constant. The derivation of these equations and the theory of light-scattering is found in reviews by Mark (1948), Oster (1948) and Doty and Edsall (1951).

TABLE.—*Effect of Increasing Concentrations of NaCl on Flocculation of Antigen-Antibody Mixtures.*

Column 3: Solution against which γ-globulin and antigen were dialysed.
Column 6: Time after adding antigen.
Column 7: A, concentration of added NaCl.
B, final Na concentration.
+ to ++++: Degrees of cloudiness from +, very faint, to ++++, heavy.
Fl.: Flocculated but not settled.
Ppt.: Precipitate settled.

Antigen.	γ-globulin solution.	Dialysing solution.	pH.	Amount of antigen added.	Time (hours or days).	Na m. equiv./l. and results.						
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
B.S.A.	P2	Distilled water	6·95	Equivalent		A	0	0·33	0·66	1·98	3·3	..
						B	0·3	0·63	0·96	2·28	3·6	..
					3 hr.		+++	++++	++++	Fl.	Fl.	..
					1 d.		Ppt.	Ppt.	Ppt.	Ppt.	Ppt.	..
„	„	„	7·6		3 hr.		++	++	++++	++++	Fl.	..
					1 d.		+++	++++	Fl.	Fl.	Ppt.	..
H.S.A.	P55	Phosphate buffer: kation 0·00013 N	6·9	„		A	0	2	4	8	12	..
					3 d.		0	+	Ppt.	Ppt.	Ppt.	..
„	P64	Phosphate buffer: kation 0·0026 N	7·6	„		A	0	0·9	2·0	4·0	..	..
						B	2·8	3·7	4·8	6·8	..	..
					4 hr.		++	+++	+++	++++	..	..
					1 d.		+++	Fl.	Fl.	Ppt.	..	..
				½	4 hr.		++	+++	+++	Fl.	..	..
				Equivalent	1 d.		+++	Fl.	Fl.	Ppt.	..	..
Ov.	P22	Distilled water	7·3	Equivalent		A	0	0·3	0·6	1·2	2·4	3·6
						B	0·4	0·7	1·0	1·6	2·8	4·0
					3 hr.		0	0	+	++	+++	++++
					1 d.		0	+	+	++	++++	Ppt.
				½ equivalent	1 d.				+++	++++	Ppt.	..
Ov.	P26	Distilled water	6·6	Equivalent		A	0	0·3	0·6	0·9	1·8	..
						B	0·4	0·7	1·0	1·3	2·2	..
					5 hr.	0	+	+	+++	++++	++++	..
					4 d.	0	+++	+++	Ppt.	Ppt.	Ppt.	..
„	„	„	7·1	„	5 hr.	0	0	0	++	+++	++++	..
					4 d.	0	++	+++	++++	Ppt.	Ppt.	..

cloudy after 3 hr. and cloudy, without precipitation, after 1 day. A slight difference can be seen between the rates of formation of ppt. by anti-Ov (P26) and antigen at pH 6·6 and 7·1.

Precipitation curves.

At low antigen/antibody ratios and low concn. of salt, sufficient to produce flocculation, the amount of ppt. was greater than found in the presence of 0·15 N Na (Fig. 2). When the amount of antigen added exceeded the equiv. the curve fell steeply to zero.

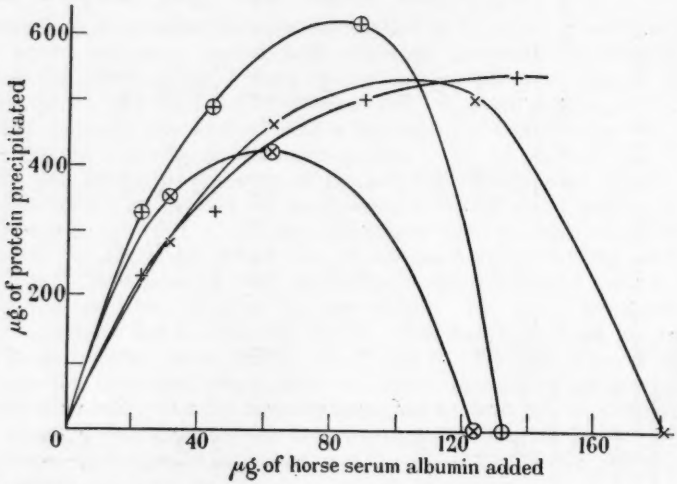

FIG. 2.—Precipitation curves of γ-globulin preparations.

+ With γ-globulin P55 pH 6·9 in 0·15 N NaCl.
⊕ With γ-globulin P55 pH 6·9 in 0·0038 N Na concentration.
× With γ-globulin P64 pH 7·6 in 0·15 N NaCl.
⊗ With γ-globulin P64 pH 7·6 in 0·0047 N Na concentration.

With a Na concentration of 0·0020 N pH 6·9 the curve was similar to that formed with 0·0038 N.

We got similar results with B.S.A. as antigen and anti-B.S.A. γ-globulin (P34), dialysed against phosphate buffer; the pH in this case was 7·0 and the Na concn. 0·0026 N. In the presence of 0·15 N Na the ppt. formed with approximately 2·5 times the equivalent amount of antigen was 0·44 mg.; this was 86 per cent of the maximum ppt. No ppt. was formed in a similar mixture of antigen and antibody in the presence of 0·0026 N Na, when it was centrifuged for 1 hr. at 3000 r.p.m., after keeping at 4° for 2 days. With less than equivalent amounts of antigen the amount of ppt. in 0·0026 N Na was greater than in 0·15 N Na.

The effect of the ratio antigen/antibody is also shown in the Table (with anti-H.S.A. P64). With an equivalent amount of antigen the salt concn. needed to produce flocculation in 4 hr. was higher than when the amount of antigen was half the equivalent.

Light-scattering.

The γ-globulin solution (P26) was dialysed against distilled water; after dialysis the gelatinous material deposited in the sac was redissolved with 0·01 N NaOH; the pH of the solution was approximately 7. The total protein concn. was 0·86 per cent and anti-Ov concn. 0·25 per cent; the Na concn., by flame photometry, was 0·002 N. Results obtained with this γ-globulin solution (P26) diluted 5 times are shown in the table. Two portions of this solution were diluted 1:100. Solution D was diluted with boiled re-distilled water to which 0·01 N HCl had been added to bring the pH to 6·2; solution F was diluted with boiled re-distilled water containing 0·9 ml. of 0·09 N NaOH per 400 ml. The

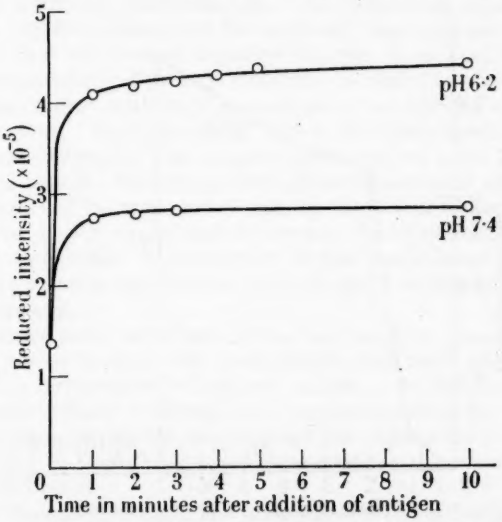

FIG. 3.—Change of reduced intensities in solutions of dialysed anti-ovalbumin γ-globulin after addition of antigen.

final pH was 7·2. The light-scattering by these solutions was measured. Owing to the high dilution, which was necessary on account of the high values found when antigen was added, the estimates of the mol. wt. of the γ-globulin in these solutions was not accurate. However, it appears that the molecules were aggregated, as the calculated mol. wt. were of the order of 300,000.

The equivalent amount (0·04 ml.) of dialysed Ov. (as determined by the optimum proportions method in the presence of 0·15 N Na) was added to each antibody solution. The light-scattering was then again measured. One min. after addition of the antigen the reduced intensity in solution D was trebled and in solution F was doubled. Fig. 3 shows the changes in the first 10 min. after the addition.

The solutions were kept at 4° and reduced intensity measurements made over a period of 7 days (Fig. 4). In solution D the intensity rose continuously and after 7 days visible floccules began to appear. In solution F the intensity remained

almost constant. When a solution of NaCl was added to F, bringing the final concn. up to 0·05 N, the turbidity rose rapidly and visible floccules were formed.

DISCUSSION.

As the reduced intensity of the γ-globulin solutions rose appreciably some degree of aggregation must have occurred on the addition of antigen, although the solutions remained clear. At pH 7·2 the reduced intensity was approximately doubled; as the antibody made up only 29 per cent of the γ-globulin the wt. of the particles in which the antibody molecules were included must have been about 4 times that of the original molecules or about 8 times that of antibody molecules that are not aggregated.

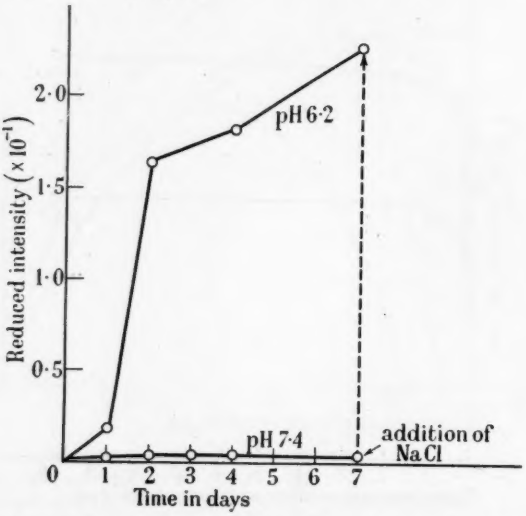

FIG. 4.—Change of reduced intensities in solutions of dialysed anti-ovalbumin λ-globulin after addition of antigen.

Since this work was finished the work of Aladjem and Liebermann (1952) on the same subject appeared. Their results appear to differ radically from ours. They found ppt. formed in mixtures of Ov. and pseudoglobulin from antiserum, when the Na concn. was minimal and that the precipitation curve in low salt concn. continued to rise far into the region of antigen excess. However, the pH of their mixtures of antigen and antibody was near 6·5, and probably fell as the ratio antigen/antibody rose owing to the low iso-electric point of ovalbumin. The difference between their work and ours in respect of the concn. of salt needed to cause precipitation illustrates the effect of pH.

It might be suggested that some of the globulin in their antibody solutions was in an unstable state at the low pH of their mixtures and was carried down with the antigen-antibody ppt. However, if this were the explanation of an increase in the amount of ppt. in low salt concn. it would be expected that it would also be carried down in the region of antibody excess, unless we can sup-

pose that the pH of the mixtures, in which antigen was in excess, was lower than that in mixtures in which antigen was not in excess. The suggestion made by Aladjem and Liebermann (1952) that the antibody solution contains molecules that can combine specifically with antigen, but are not carried down in the ppt. formed at equivalence, is interesting in view of the evidence found by Marrack, Hoch and Johns (1951) of the presence of such molecules in their γ-globulin preparations. From our study of light-scattering it appears that the formation of ppt. when the pH is near 7·0, has two stages—a certain degree of aggregation that occurs in low salt concn. and a further degree, leading to precipitation that occurs only when the salt concn. is above a certain minimum.

It is improbable that the determinants of the antigen molecules and the receptors of the antibody molecules are both negatively charged; it is also possible that the negative charges on the antibody molecule are not situated near the receptors, so that the overall negative charges of an antigen molecule may even orient an approaching antibody molecule, so that the receptor of the antibody is brought in contact with the determinant of the antigen and may therefore promote combination. The polymers, which Goldberg and Williams (1953) consider are formed, before the final stage of gel formation takes place, will present relatively few free specific combining sites (determinant and receptor) and their further aggregation will be more influenced by the overall charge. It would be expected, however, if this suggestion is correct, that the salt concn. needed to promote precipitation would be minimum at the equivalence point and would rise both in antibody and antigen excess, whereas we have found that the minimum falls in antibody excess.

Although we have no explanation of this last point we may suppose, without abandoning the lattice theory, that flocculation does take place in two stages, the first of which is independent of the salt concn.; in both stages aggregation is due to the specific affinity of receptors of antibody molecules for determinants of antigen molecules; but in the second stage the aggregates may be kept apart by the repulsion due to overall negative charges unless the effect of this repulsion is reduced by electrolytes.

The failure to flocculate even in the presence of 0·067 N NaCl, which we found in one instance, may be related to the polymerization of γ-globulin molecules detected by light scattering.

In different antigen-antibody systems, differences in the effect of reduction of salt concn. may be due to differences in the orientation of antibody mol. round an antigen mol. and to differences in the ratio of antibody to antigen mol. in the equivalence zone.

SUMMARY.

The minimum concentrations of sodium needed to cause flocculation in mixtures of antigen and antibody have been measured.

The minimum varies in different preparations of antigen and antibody.

The minimum depends on the pH of the mixture.

The precipitation curve in low salt concentrations differs from that in the salt concentrations conventionally used.

From light-scattering measurements it appears that antigen and antibody molecules combine in low concentrations of salt although the solutions remain clear.

Our thanks are due to Dr. J. R. K. Preedy for estimations of sodium concentrations, to Mr. R. G. S. Johns for estimations of pH and of proteins, and to Dr. E. Orlans for estimations of proteins; also to Dr. P. Johnson of the Department of Colloid Science, Cambridge, for advice on the measurement of light-scattering.

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THE EFFECT OF CORTISONE ON MACROPHAGE ACTIVITY IN MICE.

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IN an earlier study of the tuberculin reaction in rabbits (Gell and Hinde, 1951) we had concluded that its inhibition by cortisone was due, at the cellular level, to a failure of immigration of reticulo-endothelial mononuclear cells, a non-specific effect unrelated to the immune nature of the reaction. Mononuclear cells already present at the site of the reaction, and the few that did enter it, appeared to respond as in the normal animal. Has cortisone then a direct action on the mononuclear cells, inhibiting their power of movement and possibly other activities, or is their failure to migrate due to changes in the environment—either an alteration in the tissues setting up a barrier to cell movement, or a suppression of the usual stimulus to cell movement?

An indirect attack was made on this problem by investigating phagocytosis by reticulo-endothelial cells, to see whether we could demonstrate any direct action by cortisone on this cell function. In order to isolate the phagocytic function, experiments were carried out using macrophages in two anatomical situations, in both of which phagocytosis is independent of cell movement.

I. The activity of the fixed macrophages responsible for clearing the blood of foreign material was studied by measuring the rate of uptake of colloidal radioactive gold from the circulation. Mice were given 5–25 μg. gold intravenously (4–12 μC. activity) in 0·1 ml. and samples of blood were removed at 1½, 2, 2½ and 4 minutes for estimation of radioactivity.

Table I summarises the results of three experiments. The uptake is, as might be expected, approximately logarithmic, therefore the log percentage of radioactivity remaining in the circulation at each time interval is tabulated, taking the one minute value as 100 per cent. At no time do the differences between the test and control animals approach significance. The activity of the various organs (liver, spleen, lung, kidney and carcass) three hours after the injection was also estimated and no differences in the differential uptake were demonstrable. In all cases about 90 per cent of the activity was in the liver.

II. The phagocytosis by peritoneal macrophages of a formolised suspension of *Staphylococcus albus* (10^9 organisms in 0·5 ml.) was measured by counting the number of ingested bacteria under the microscope. The macrophage exudate

TABLE I.—*Uptake of Intravenous ^{197}Au : Mean Logs of Percentages of One-Minute Values.*

Experiment	I (8 mice).				II (10 mice).				III (10 mice).			
Time (min.)	1½.	2.	2½.	4.	1½.	2.	2½.	4.	1½.	2.	2½.	4.
Without cortisone	1·80	1·63	1·51	1·14	1·90	1·84	1·81	1·63	1·87	1·80	1·70	1·42
With cortisone	1·86	1·68	1·58	1·26	1·94	1·89	1·84	1·67	1·86	1·73	1·62	1·38
Difference	−·06	−·05	−·07	−·12	−·04	−·05	−·03	−·04	+·01	+·07	+·08	+·04

was produced by the intraperitoneal injection of a mixture of equal parts of 40 per cent Lemco and 20 per cent gum acacia three days before the experiment. In the peritoneal smears taken ten minutes after the injection of bacteria, organisms were only seen in typical foamy macrophages and the contents of 200 of these cells were recorded.

Previous experiments had shown that we could ignore the absolute macrophage count, the excess of bacteria being such that any variations in the absolute number of macrophages did not affect significantly the rate of phagocytosis.

The block-diagram in the Figure shows the results of three experiments using in each 6 mice, of which 3 received 1–3 mg. cortisone 4 hours, or 18 and 4 hours,

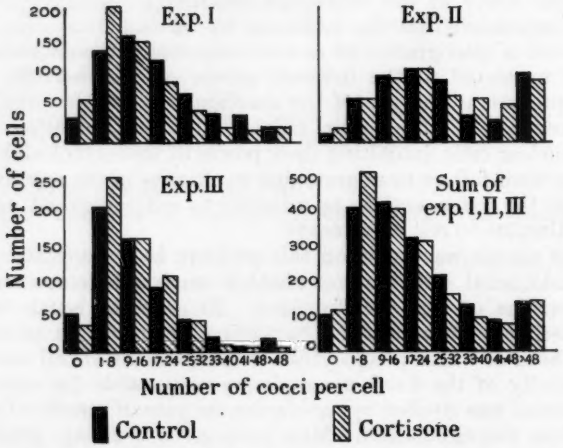

FIGURE.—Phagocytosis of cocci by macrophages of peritoneal exudate.

before the experiment. The pattern of distribution of the number of bacteria ingested by individual cells was very consistent in all 6 mice of any one experiment; in none of the experiments did treatment with cortisone alter this pattern. Comparison of the total number of bacteria contained in 200 macrophages by application of the *t*-test to (*i*) the mean number of bacteria per mouse ($p = 0{\cdot}6$–$0{\cdot}8$), and (*ii*) the mean number of bacteria per group in each experiment ($p = 0{\cdot}6$–$0{\cdot}7$) (see Table II), gives evidence for the purely random distribution of these results between test and control groups, and therefore suggests that cortisone has had no effect either on the number of macrophages ingesting bacteria or on the activity of individual macrophages.

DISCUSSION.

Both approaches therefore lead to the conclusion that cortisone does not interfere directly with the function of macrophages, at least in the two situations in which we have studied them. Several other workers have investigated the relation of the adrenal cortex to the phagocytic activity of the reticulo-endothelial cells. Our results on the uptake of colloidal gold may be compared with those of Germuth, Ottinger and Oyama (1952) using bacteria. They confirmed the findings of White and Marshall (1951) that the blood clearance of virulent bacteria

TABLE II.—*Number of Bacteria Ingested by Cells of Control and Cortisone-treated Mice.*

	Control: number in 200 cells.	Total (3).	Cortisone: number in 200 cells.	Total (3).	Difference.
I	3663, 3767, 3407	10836	2642, 2241, 3465	8348	+1488
II	4987, 6182, 5994	17163	4264, 7310, 6762	18336	−1173
III	2775, 1852, 3105	7782	2014, 2375, 2635	7024	+ 758
Mean/mouse		3970±487		3745±664·0	Mean difference/experiment, 358±793·5; $p = 0·6$–$0·7$
Difference between means, 225±820; $p = 0·7$–$0·8$					

was unaffected by cortisone in the first two to three hours, though subsequently a severe bacteriaemia developed in treated animals, and found further that the clearance of avirulent bacteria was completely unaffected by cortisone, suggesting that the result with virulent organisms was not due to any failure of the blood-clearing mechanism.

Phagocytosis in the peritoneal cavity was studied by Spain, Molomut and Haber (1950), using conditions where movement of macrophages was required both to approach and to remove injected carbon particles; they concluded that cortisone had an inhibitory effect. However,we ourselves deliberately chose conditions under which such movement is not required, and in view of our results it is likely that the effect found by these authors was due to inhibition of cell movement rather than of phagocytosis as such.

Our results are consistent with the theory that cortisone suppresses mesenchyme cell activity in tissues so strikingly by altering the tissue environment, not the cells themselves; the sensitivity of this function to environmental differences is here illustrated by the marked variation, in rate or pattern of phagocytosis between individual experiments of both groups. Indeed there is evidence that adrenocortical activity does produce changes in tissues, such as a reduction in tissue permeability (Opsahl, 1949) and in the permeability of the capillary wall to leucocytes (Ebert and Wissler, 1951) which may well interfere with the movement of cells.

The small amounts of radioactive gold required were kindly supplied by Dr. W. K. Sinclair of the National Cancer Hospital, to whom our thanks are due. The cortisone was provided from the gift made by Merck & Co. Inc. jointly to the Medical Research Council and the Nuffield Foundation.

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CHEMOTAXIS OF MONOCYTES.

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ALTHOUGH Clark, Clark and Rex (1936) observed that macrophages† in the transparent tail of the tadpole migrated towards areas of injury, and also towards egg albumen, starch and croton oil, chemotaxis has not been demonstrated unequivocally for the macrophage *in vitro*, as it has been for the granulocyte. Chambers and Fell (1931) noticed that chick embryo macrophages in tissue culture collected about cells which had been injured by micromanipulation, and a similar observation was made by Jacoby (1937) with monocytes obtained from hen blood. Lasfargues (1946), studying the migration of macrophages from explants of guinea-pig spleen in plasma coagula, noticed in one preparation which had been accidentally contaminated that the macrophage migration zone was larger on the side of the explant where the colony of contaminant bacteria had grown. The organism responsible was shown to be a non-pathogenic Gram-positive coccus. Other non-pathogenic bacteria, however, both Gram-positive and Gram-negative, produced no effect on the migration zone of the macrophages, and pathogenic staphylococci exerted a toxic effect on the cells. Coman (1940), using explants of rat omentum, was unable to show that *Mycobacterium tuberculosis* or *Staphylococcus aureus* produced any change in the migration zone of macrophages, although granulocytes under the same conditions were strongly attracted by these organisms. McCutcheon (1946), reviewing the data on chemotaxis in leucocytes, summed up the position in the following way: "The question as regards chemotaxis in monocytes is not completely answered. From present evidence it seems likely that chemotaxis plays a smaller part in the activity of these cells than it does in polymorphonuclear leucocytes."

In the present investigation the photomicrographic trace technique previously described for demonstrating chemotaxis in granulocytes (Harris, 1953) was modified for the study of monocytes. With this technique the question of chemotaxis in monocytes was re-examined.

EXPERIMENTAL.

The technique for maintaining mammalian monocytes *in vitro* has been described in detail by Mackaness (1952). The monocytes were obtained by injecting paraffin oil into the peritoneal cavity of a rabbit, and washing out the resulting exudate after a period of six days. The cells were suspended at a suitable density in Hanks' solution (Hanks, 1948) containing 20 per cent of aged rabbit serum, and were introduced into specially prepared slide-cell chambers. The chambers devised by Mackaness were modified in the present experiments by the removal of the centre well, so that the object to be tested could be inserted when required. In these modified chambers the cells could be kept alive for many days at 37° by periodic changes of the medium. The test-object was inserted into the chamber through one of the lateral drill-holes by means of a hypodermic needle, generally between the 7th and the 10th day of incubation.

The movements of the monocytes relative to the test-object were recorded photographi-

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† In this paper the terms "monocyte" and "macrophage" are used synonymously.

cally by continuous exposure under dark ground illumination. The details of this technique as used for studying chemotaxis in granulocytes, have been described elsewhere (Harris, 1953). The optical system for recording monocyte movement was the same as that used for granulocytes, and Kodak " Microfile Pan " film was found to be suitable in both cases. Since monocytes move much more slowly than granulocytes, it was found necessary in the present experiments to expose each negative for 45–60 min., instead of for the 5–15 min. required for granulocytes. The intensity of the illumination was correspondingly reduced.

RESULTS.

Random movement.

When the suspension of monocytes was introduced into the chamber the cells settled on the bottom coverslip, and at 37° soon became closely adherent to the glass surface. While adherent in this way the monocytes showed very little motility, although they spread out on the glass, formed pseudopodia, and assumed amoeboid forms. Photographic records of the cells at this stage confirmed that there was negligible change of position. The period of close adhesion to the glass sometimes lasted as long as three or four days, and the introduction of a test-object at any time during this period produced no visible effect. Eventually, however, the cells became actively motile, although they remained loosely adherent to the coverslip. Under optimal conditions they moved at about one-quarter the speed of granulocytes. The test-object was not inserted into the chamber before the seventh day, although the monocytes were usually motile well before this time. A small percentage of granulocytes is always present in the exudate obtained from the rabbit peritoneal cavity by the present method, but by the seventh day these had died and had been ingested by the monocytes. Any lymphocytes originally present in the preparation were washed out by the periodic changes of medium, since, unlike monocytes and granulocytes, they did not adhere to the glass. A pure population of monocytes therefore remained. Fig. 1 shows a sample of the cells finally present in the chambers ; they are all typical monocytes. The type of pattern traced out by the monocytes when moving freely at random is shown in Fig. 2. It will be seen that the traces are tortuous, irregular and of varying width, indicating that the cells moved in a haphazard way, at different speeds, and in a frequently changing direction. The traces produced by the monocytes were denser and less tortuous than those produced by granulocytes under similar conditions, since the monocytes moved more slowly and changed direction less frequently.

The effect of micro-organisms.

If a clump of *Staphylococcus albus* was allowed to dry on a slide and was then inserted into a chamber in which the monocytes were moving freely, all the cells within a certain range of the clump moved directly towards it. As the monocytes made contact with the bacteria, their migration almost ceased, and phagocytosis began. In this way an accumulation of phagocytes was produced in and around the clump. When all the bacteria had been ingested, the cells gradually dispersed and resumed random migration. Similar results were obtained with clumps of *Mycobacterium tuberculosis* (H37Ra), *Erysipelothrix monocytogenes*, *Corynebacterium diphtheriae*, *Salmonella typhi*, *Staphylococcus aureus*, *Streptococcus pyogenes*, the Weybridge strain of *Bacillus anthracis*, and with starch grains. Fig. 3 shows the pattern traced out by the monocytes in the presence of a clump

of *Myco. tuberculosis*. It will be seen that all the traces within a certain range of the clump converge directly upon it. These traces, besides being denser than those produced by granulocytes under similar conditions, are considerably straighter, thus indicating that the movement of the monocytes in response to a chemotactic stimulus is even more direct than that of the granulocytes.

With the exception of the *Staph. aureus* and the *Str. pyogenes*, all the organisms listed above had been found to be chemotactic for granulocytes. The *Staph. aureus* and the *Str. pyogenes*, being leukocidin-producing strains, killed all the granulocytes within a certain radius, so that in the plasma film preparation used for granulocytes neither chemotaxis nor phagocytosis could be demonstrated with these two organisms. In the monocyte preparations it was not possible to test accurately the capsulated strains of *B. anthracis* and *Bact. friedländeri*, which had been shown not to be chemotactic for granulocytes. When a clump of these organisms was inserted into the chamber, some spreading of the mucinous capsular material invariably occurred, so that the clump did not have a sharply defined edge. Under such conditions failure to demonstrate polarization of the monocyte traces in the vicinity of the clump could not be considered conclusive; it was, however, significant that the accumulation of monocytes which occurred around the clumps of chemotactic bacteria could not be observed around these organisms.

The effect of autolysed tissues and tissues partially digested by pepsin and by trypsin.

Fragments of rabbit muscle and liver which had been allowed to autolyse under aseptic conditions for from 3–24 hours, and others which had been partially digested with pepsin or trypsin, were inserted into the chambers. These tissue fragments were not chemotactic for the monocytes. Fig. 4 shows the movement of the monocytes in the presence of a fragment of autolysed muscle; the cells in the vicinity of the tissue have moved in an entirely random way. Although records were taken continuously for many hours, and, on several occasions, every day for 3 or 4 days, no evidence of polarization of the monocyte traces in the vicinity of the tissue fragments was observed. Some of the monocytes, which during random migration had come into contact with the fragments, adhered to them, as they do to most débris in the preparation; but there was never a rapid accumulation of phagocytes, such as occurred within an hour or two around a clump of chemotactic bacteria.

DISCUSSION.

The present investigations show clearly that mammalian monocytes are no less subject to chemotaxis than granulocytes. Like the latter, monocytes move in

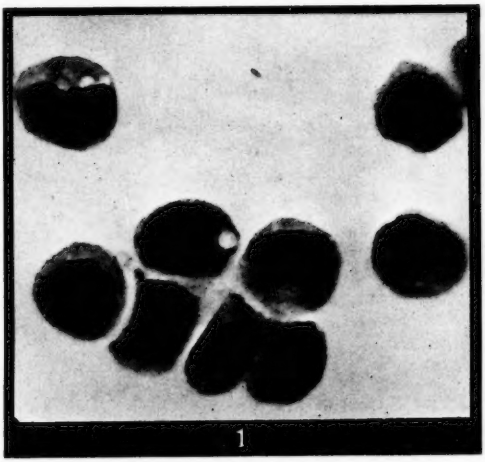
DESCRIPTION OF PLATES.

FIG. 1.—A sample of the cells finally present in the chambers; they are all typical monocytes. (Giemsa; × 760.)

FIG. 2.—The pattern traced out by the monocytes, when moving at random. (Exposure 1 hour; × 360).

FIG. 3.—The pattern traced out by the monocytes in the presence of a clump of *Mycobacterium tuberculosis* (H37Ra); it will be seen that all the traces within a certain range of the clump converge directly upon it. (Exposure 1 hour; × 360.)

FIG. 4.—The pattern traced out by the monocytes in the presence of a fragment of autolysed muscle; the cells in the vicinity of the fragment have moved in an entirely random way. (Exposure 45 mins.; × 360.)



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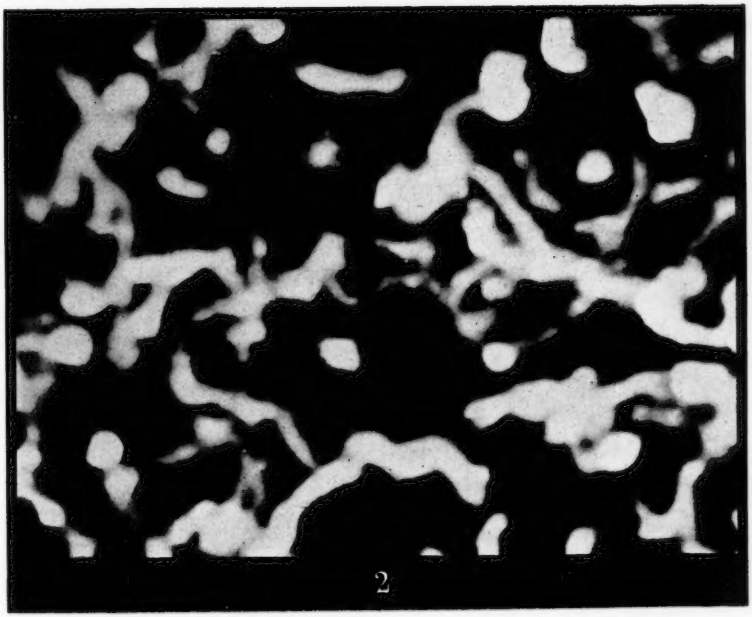

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Harris.

direct lines towards a clump of chemotactic bacteria, accumulate in and around the clump and eventually completely ingest it. The reactions of the two cells to chemotactic stimuli thus differ only in that the sequence of events takes place more slowly in monocytes. Futhermore, those species of bacteria and starch grains which had been shown to be chemotactic for granulocytes, were found to be chemotactic also for monocytes, while tissue breakdown products were chemotactic for neither. The two capsulated organisms (*B. anthracis* and *Bact. friedländeri*) that had no effect on granulocytes could not be tested accurately in the monocyte preparations owing to spreading of the mucinous capsular material; and two others (*Staph. aureus* and *Str. pyogenes*) which attracted the monocytes were toxic to the cells under the conditions of the granulocyte experiments. In all the valid tests, however, monocytes and granulocytes reacted or failed to react chemotactically to the same stimuli. The view that certain bacteria are associated with a predominantly monocyte tissue reaction because they produce some substance specifically chemotactic for monocytes, whereas others are associated with a predominantly granulocyte reaction because they produce a substance specifically chemotactic for granulocytes, must therefore be seriously questioned. It would appear rather that chemotaxis is a non-specific reaction to which both monocytes and granulocytes are subject in response to certain stimuli; whether one cell or the other predominates in any given lesion must be determined by other factors.

SUMMARY.

A method previously described for demonstrating chemotaxis in granulocytes was modified for the study of chemotaxis in monocytes obtained from the rabbit peritoneal cavity.

By this method it was shown that monocytes were no less subject to chemotaxis than granulocytes. Although the movement of the monocytes was slower, their advance towards a chemotactic object was, if anything, more direct than that of granulocytes.

Within the limits of the experiments monocytes reacted chemotactically to the same stimuli as granulocytes.

Staph. albus, *Staph. aureus*, *Str. pyogenes*, *Myco. tuberculosis*, *Ery. monocytogenes*, *C. diphtheriae*, *Salm. typhi*, *B. anthracis* and starch grains were chemotactic for monocytes; products of the breakdown of tissues were not.

It is a pleasure to thank Sir Howard Florey for his help in this work. I am indebted also to Mr. A. A. Argyle for technical assistance, and Mr. B. H. Glass for the photography.

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THE EFFECT OF BILE DUCT OBSTRUCTION ON REGENERATION OF THE RAT'S LIVER.

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TOTAL obstruction to the outflow of bile is known to produce considerable changes in the liver. Proliferation of bile ducts, increase in supporting connective tissue and parenchymal changes have been described in many experimental animals, including guinea-pigs (McMahon, Lawrence and Maddock, 1929), rats (Cameron and Oakley, 1932) and cats (Stewart and Lieber, 1934). Functional disturbances have been shown to occur (Edlund, 1948; Koch-Weser, Meyer, Yesenick and Popper, 1952) and several workers have concluded that the regenerative capacity of the parenchymal cell is lost or severely reduced in biliary obstruction (Mann, Fishback, Gay and Green, 1931; Higgins and Anderson, 1932; Cameron, 1935; Edlund and Holmgren, 1945; Edlund, 1948; Himsworth, 1950; Payling Wright, 1951).

Mann and his colleagues emphasized that obstruction of the common bile duct is one of the factors which tend to prevent restoration of the partially resected liver in experimental animals. In many patients with prolonged obstructive jaundice, the absence of striking nodular regeneration is often attributed to a diminished capacity of the parenchymal cells to multiply (Lieber and Stewart, 1934; Sherlock, 1952). Examples of the development of regeneration nodules and Laennec's cirrhosis following total bile duct obstruction have, however, occasionally been reported (Frerichs, 1861; Gibson and Robertson, 1939; Lichtman, 1949). Moon (1934) and Dible (1951) indicated that this development may occur in obstructive jaundice and Love (1932) showed mitoses and apparent recovery and regeneration of parenchymal cells after experimental phosphorus poisoning in animals with common bile duct obstruction. The experiments recorded in this paper were carried out in order to test the regenerative capacity of liver parenchymal cells after partial hepatectomy, in the presence of biliary obstruction.

EXPERIMENTAL.

Female albino rats weighing 100–260 g. were kept on a diet consisting of "Research" rat cubes supplemented with white bread and greens. The rats were not fasted pre-operatively or before being killed.

The following experiments were designed to compare the growth of the residual lobes of the liver after partial hepatectomy in normal animals and in animals after varying periods of bile duct obstruction.

Group 1.—*Partial hepatectomy in normal animals.*

Removal of the anterior lobes (median and left lateral) approximately $\frac{2}{3}$ of the total liver mass, was carried out under ether anaesthesia in 21 rats after the method of Higgins and Anderson (1931), special care being taken to divide the hepatic ligaments before ligation of the pedicles to avoid accidental ligation of veins (Doniach and Weinbren, 1952). Groups

* In receipt of a grant from the Medical Research Council.

of 3 or more rats were killed at varying times between 3 and 21 days later and the residual liver lobes weighed. In 12 control rats killed immediately after the operation the ratio of the total liver wt. to the wt. of resected anterior lobes was 1·54 ± S.D. 0·087.

Group 2.—Bile duct ligation and division.

The method described by Cameron and Oakley (1932) was used and in 40 rats the common bile duct was divided at a point about half-way between the junction of the two hepatic ducts and the duodenum. Animals were killed at intervals from 3 to 36 days after operation. After removal, the livers were inspected for the presence of necroses and the distended extrahepatic bile ducts were measured, emptied of their contents and excised. The median and left lateral lobes were weighed separately from the right and omental lobes. This was done in order to determine if the anterior liver lobes maintained a constant weight relationship to the posterior lobes after bile duct ligation.

Group 3.—Combined bile duct ligation and partial hepatectomy.

An operation combining the two above procedures was performed in 21 rats. They were killed in groups of 3 or more after 3–21 days.

Group 4.—Partial hepatectomy in the presence of established biliary obstruction.

In this series of 30 rats bile duct ligation was carried out and at varying times from 3–21 days later $\frac{2}{3}$ partial hepatectomy was performed. In many cases, when the bile duct was grossly dilated the usual technique had to be modified so that the median and left lateral lobes were removed separately. To reduce the risk of haemorrhage 2 mg. of a vitamin K analogue were injected intramuscularly 30–60 min. before operation. The animals were killed 3 days after partial hepatectomy.

All animals were killed by ether and those which were not visibly jaundiced in series 2, 3 and 4 were discarded from the main data.

A specimen of heart blood for serum bilirubin estimation was taken from each rat in these groups before death to confirm the presence of icterus. Distended extrahepatic bile ducts were emptied and excised before weighing, wet and dry weights were measured and a specimen taken for histology in each case.

Dry weights.—These were estimated after 4–7 days in an oven thermostatically controlled at 105°. As a preliminary measure, 10 g. of normal liver tissue and 10 g. of liver tissue after 12 days of biliary obstruction were placed in this oven, and the daily weights were charted. It was found that considerable weight loss took place during the first 3 days, after which time the loss was insignificant.

The usual fixative was Helly's fluid and occasionally 6 per cent formol saline was used.

Estimation of relative proportions of parenchymal tissue and bile ducts.—In certain cases it was necessary to determine this relationship, and sections stained with phosphotungstic acid haematoxylin were found to show a very good contrast between parenchyma and bile ducts. A method similar to that described by Ogilvie (1937) to estimate pancreatic islet tissue, was used and sections were projected at a magnification of × 45 on squared paper, 40 cm. distant from the ocular. Liver cell tissue and bile ducts were traced out in 10 fields of area 316 sq. μ. in the depths and on the edge of the section. The paper was cut up and pieces representing the areas of different components were weighed separately.

The relationships of parenchymal tissue and bile duct areas were compared in the resected lobes and in the residual lobes 3 days after partial hepatectomy in the same animal. Lobes with a high proportion of bile ducts did not show a marked difference in sp. gr. from lobes with a high proportion of parenchymal tissue and the area relationships were therefore regarded as proportional to wt. relationships of the two components. Specific gravity was estimated from the formula :

$$\text{Sp. gr.} = \frac{\text{Wt. in air (w)}}{\text{Wt. in air} - \text{wt. in water (v)}}.$$

RESULTS.

Table I shows that the ratio of total liver wt. to the wt. of left lateral and median lobes proved to be fairly constant in both the normal rats and the rats

TABLE I.—*Relationship of Weight of Anterior Lobes to Weight of Total Liver.*

Days following bile duct ligation.	No. of rats.	Total liver / Anterior lobes	S.E. of mean.
Normal	12	1·54	±0·038
1–6	7	1·55	±0·032
7–12	4	1·61	±0·051
13–18	4	1·61	±0·18
19–24	7	1·57	±0·30
25–36	4	1·54	±0·11
Total and mean of whole series	38	1·56 (S.D. ± 0·10)	±0·016

with biliary obstruction. The mean value, 1·56 (S.D. ± 0·1) was used to estimate total liver wt. in all the experiments involving partial hepatectomy. In such cases the weights of the liver remnants were expressed as percentages of total estimated liver weights. This expression of hepatic increment is known to be more accurate than values which involve a liver wt./body wt. relationship (Brues, Drury and Brues, 1936), and is preferred by most subsequent workers (Harkness, 1952). The final percentage was corrected in each case to allow for loss or gain in body wt. as a result of operation trauma or normal growth (Brues *et al.*, 1936). This was considered necessary in view of the well-recognised lability of liver wt. in fasting states (Addis, Poo and Lew, 1936).

Partial hepatectomy.

The residual lobes showed an increase in wt. similar to that found by previous workers and the remnant after 3 days weighed 75·57 ± S.D. 5·4 per cent of the estimated total liver wt.

In Table II, the detailed weights have been listed. The final percentage of liver increment in all the relevant experiments were derived in the way indicated in this table.

TABLE II.—*Increase in Weight of Liver 3 Days after Partial Hepatectomy.*

Rat.	Body wt. at death in g.	Resected liver in g.	Estimated total liver in g.	Residual liver after 72 hr. in g.	Residual liver / Estimated total liver. × 100	After correction for change in body wt.
a	150	4·20	6·55	4·87	72·90	77·76
b	175	3·87	6·04	4·42	73·17	73·17
c	140	4·49	7·60	5·65	78·59	81·4
d	145	4·62	7·21	5·54	76·90	76·9
e	145	4·32	6·74	4·50	67·96	69·1

$m = 75{\cdot}57 \pm 5{\cdot}4$ (S.D.) per cent.

Increase in weight of liver after simple bile duct obstruction.

In this series liver wt. could only be expressed as a percentage of body wt. Table III shows that the wt. of the liver increases considerably after bile duct obstruction ; water content (Table IV) is slightly increased, but this latter cannot account for the increased wt. of the liver. Sections show a characteristic proliferation of bile ducts mainly of the perilobular type and many mitotic figures

TABLE III.—*Increase in Weight of Liver after Bile Duct Ligation.*

Days following bile duct ligation.	Number of rats.	Liver wt. / Body wt. × 100	S.E. of mean.
0	12	3·47	±0·038
6	3	5·94	±0·42
12	4	7·24	±0·58
18	3	8·39	±0·61
24	4	7·21	±0·68
30–36	2	8·65	—

TABLE IV.—*Water Content of Livers after Bile Duct Ligation.*

Days following bile duct ligation.	Number of rats.	Water content in per cent.	S.E. of mean.
0	4	71·51	±0·16
6	3	75·8	±1·66
12	7	76·56	±0·39
18	5	75·59	±1·13
21	8	75·78	±0·62
24	3	76·46	±0·72

are found in bile duct cells as late as 36 days post-operatively and occasionally in parenchymal cells during the first 18–20 days. There is thus an increase of solid material in the livers in this series after bile duct ligation and this increment probably reflects mainly the well-marked proliferation of bile duct tissue. This finding confirms the opinion of Edlund who suggested that the increment after biliary obstruction is induced, is caused by proliferating bile duct tissue and increase in water content.

Increase in weight of liver after combined bile duct obstruction and partial hepatectomy.

After this procedure the remnant enlarges rapidly so that after 20 days its wt. is equivalent to that of a whole liver, the bile duct of which has been obstructed for a comparable period of time. Liver remnants weighed 7·97 per cent of body wt. 18–21 days after the combined operation and total liver in cases of bile duct obstruction only weighed 8·39 and 7·21 per cent of body wt. after 18 and 24 days respectively (Tables III and IV).

Table V demonstrates the striking difference between the weights of the residual lobes in this group and those of the corresponding posterior lobes in

TABLE V.—*Comparison of Weights of Posterior Lobes after Bile Duct Obstruction Alone or Combined with Partial Hepatectomy*

Days after ligation.	Simple bile duct obstruction.			Combined bile duct obstruction and partial hepatectomy.		
	Number of rats.	Posterior lobe / Body wt. × 100	S.E. of mean.	Number rats.	Posterior lobe / Body wt. × 100	S.E. of mean.
0	12	1·28	±0·087.	—	—	—
3–6	5	1·92	±0·13	11	3·59	±0·21
12–15	7	2·50	±0·29	3	5·56	±0·30
18–21	7	2·90	±0·32	4	7·97	±1·18

rats with simple bile duct obstruction and no partial hepatectomy. It is of interest that the most rapid growth takes place during the first 3 days and the curve parallels that shown after partial hepatectomy in otherwise normal rats (Fig. 1*a*, 2).

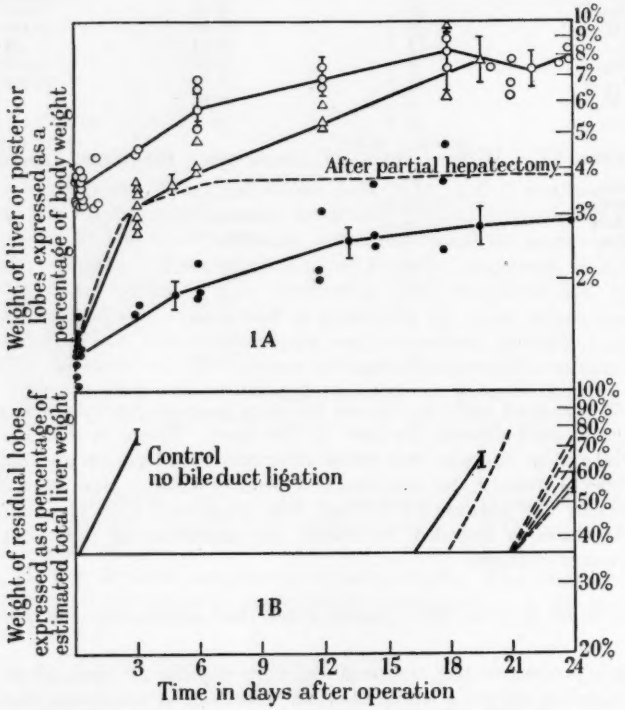

Weight of liver or posterior lobes expressed as a percentage of body weight
After partial hepatectomy
1A
Weight of residual lobes expressed as a percentage of estimated total liver weight
Control
no bile duct ligation
1B
Time in days after operation

FIG. 1.—Graphs showing alteration in wt. of total and residual liver after different operative procedures.

FIG. 1*a*.—Increase in moist wt. of total liver and posterior lobes after bile duct ligation and of residual lobes after combined bile duct ligation and partial hepatectomy.

○ Total liver after bile duct ligation.
● Posterior lobes after bile duct ligation.
△ Residual lobes after bile duct ligation and partial hepatectomy.

FIG. 1*b*.—Shows increase in wt. of residual liver following partial hepatectomy after varying periods of bile duct obstruction. The dotted lines represent the estimated increase in wt. of parenchymatous tissue. The vertical lines represent + and − the standard error of the mean value.

———— residual liver.
– – – – – – parenchyma.

Bile duct obstruction and subsequent partial hepatectomy.

In rats with established biliary obstruction, the remnant 3 days after partial hepatectomy reached a greater weight than the posterior lobes in control cases with bile duct obstruction only. Expressing the wt. of the residual lobes as a

TABLE VI.—*Percentage Increment of Liver on the 3rd Day following Partial Hepatectomy at Varying Times after Bile Duct Ligation.*

Days after ligation.	Number of rats.	$\frac{\text{Residual lobes}}{\text{Estimated total}} \times 100$	S.E. of mean.
Control (0)	5	75·67	±2·01
15–18	6	66·82	±2·79
21	4	57·09	±2·69

percentage of the estimated total liver wt. at the time of partial hepatectomy and making corrections for wt. gain or loss of the animal, the values are however, slightly lower than that found in otherwise normal partially hepatectomized rats (57·09 per cent at 21 days as opposed to 75·67 per cent in controls, Table VI).

The values of the actual increment of liver mass were roughly equivalent, as the average increase in wt. of the residual livers was 2·64 g. at 21 days, 2·91 g. at 15–18 days and 2·45 g. in controls.

As can be seen from Fig. 3, the livers in cases of biliary obstruction show a well-marked increase in the ratio of bile duct tissue to parenchyma when compared with normal livers. This in turn produces an increased total liver wt., as has been shown in Table III. The increment in wt. of the residual lobes after hepatectomy, therefore, although roughly equivalent in absolute value to that in normal rats, appears lower when expressed as a percentage of the estimated total wt. at the time of resection.

Table VII shows that the ratio of parenchymal tissue to bile duct tissue is increased after partial hepatectomy in each of the cases studied by the projection method.

TABLE VII.—*Proportions of Parenchymal Tissue and Bile Duct Tissue derived from Measurements of Areas after Projection.*

Rat.	Days after ligation.	Parenchyma/Bile-duct ratio in resection.		Parenchyma/Bile-duct ratio in residuum.	
		(P./BD.).	(per cent parenchyma).	(P./BD.).	(3 days later) (per cent parenchyma).
f	21	1·96	66	2·40	71
g	21	2·62	72	5·46	85
h	21	1·27	56	2·74	73
i	21	2·19	69	2·73	73
j	18	2·29	70	2·81	74

The values listed in Table VII may be used to give an estimate of the probable total mass of parenchymal tissue in the resected and residual lobes after the 3-day period in the same rat. The results are regarded as only approximate as the method did not permit separation of hepatic cells from connective tissue and endothelial cells within the lobule, and all these elements have been included with the parenchymal tissue as distinct from bile duct tissue.

In Table VIII parenchymal tissue increment has been estimated in 5 rats in which partial hepatectomy was performed after prolonged biliary obstruction. It is seen that by using this method the values now agree with the expected increment of liver after partial hepatectomy in otherwise normal rats. Histological findings are confirmatory in this respect and a striking increase in parenchy-

TABLE VIII.—*Increment after Partial Hepatectomy expressed as a Percentage of Estimated Total Parenchymal Tissue in Rats with Biliary Obstruction.*

Rat.	Resected in g.	Estimated total liver in g.	Parenchyma in total in g.	Residuum in g.	Parenchyma in residuum in g.	$\frac{\text{Residuum parenchyma}}{\text{Total parenchyma}} \times 100$	Correction for body wt. change in per cent.
f	7·47	11·65	7·69	6·47	4·59	59·68	63·46
g	8·97	13·99	10·07	8·52	7·24	71·91	76·60
h	10·90	17·00	9·52	8·40	6·13	64·39	71·54
i	9·86	15·38	10·61	7·97	5·82	54·85	54·85
j	7·74	12·07	8·45	8·04	5·95	70·41	77·90

mal mitotic figures is seen in all residual lobes on the 3rd day following partial hepatectomy after even 21 days of biliary obstruction (Fig. 3, 4, 5 and 6). The lobules which in this series of experiments are demarcated by proliferating bile ducts, show a well-marked increase in diameter.

The main findings have been summarized in Fig. 1. In the upper graph (Fig. 1*a*), the points represent values of liver weights expressed as a percentage of body wt., plotted on semilogarithmic graph paper.

The lower graph (Fig. 1*b*) represents growth of residual liver after partial hepatectomy in rats with and without biliary obstruction. The points indicate values of liver remnants expressed as a percentage of total liver wt. at the time of operation, as estimated from the weights of resected lobes, also plotted on semilogarithmic graph paper (Brues *et al.*, 1936).

Rats with ascites.—After operations involving the common bile duct 9 rats became ill and developed moderate to severe ascites. Four in this group died 3 or 4 days after operation and 5 survived for as long as the healthier animals in the same series. At post mortem examination, the abdomen contained up to 125 ml. bile-stained fluid, the common bile duct was usually not distended and the liver did not appear grossly abnormal. In 3 cases with severe ascites the liver was distinctly smaller than in unoperated controls and the lobes were deformed and atrophied. In 2 rats with moderate ascites the liver weights were lower than those found in healthy operated animals but greater than those found in untreated controls. These findings were seen both in cases in which ascites followed bile duct ligation only and in bile duct ligation combined with partial hepatectomy. The most striking histological feature of the liver in cases of

EXPLANATION OF PLATES.

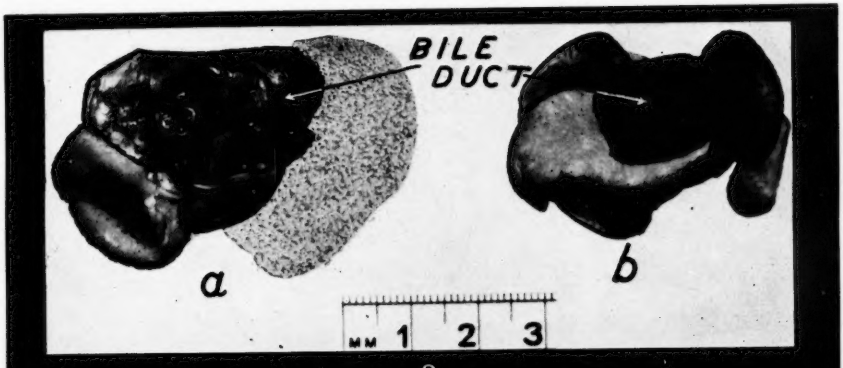
FIG. 2.—Posterior view of (*a*) total liver 25 days after bile duct ligation and (*b*) residual liver 19 days after combined bile duct ligation and partial hepatectomy. The anterior lobes in the left-hand specimen have been stippled and the dilated bile ducts and posterior lobes have been outlined. The residual lobes of specimen (*b*) are much larger than the corresponding lobes of specimen (*a*) and, in fact, are equivalent in wt. to the total liver.

FIG. 3.—Section of liver resected from rat 20 days after bile duct ligation; phosphotungstic acid haematoxylin: shows darkly-stained parenchyma and pale bile-duct tissue, × 45.

FIG. 4.—Section of residual liver 3 days after partial hepatectomy taken from the same rat as Fig. 3; phosphotungstic acid haematoxylin: shows the relative increase in parenchymal tissue, × 45.

FIG. 5.—High power view of same section as Fig. 3; phosphotungstic acid haematoxylin: shows proliferating bile ducts, × 900.

FIG. 6.—High power view of same section as Fig. 4; phosphotungstic acid haematoxylin: shows numerous mitotic figures in the parenchymal cells, × 430.



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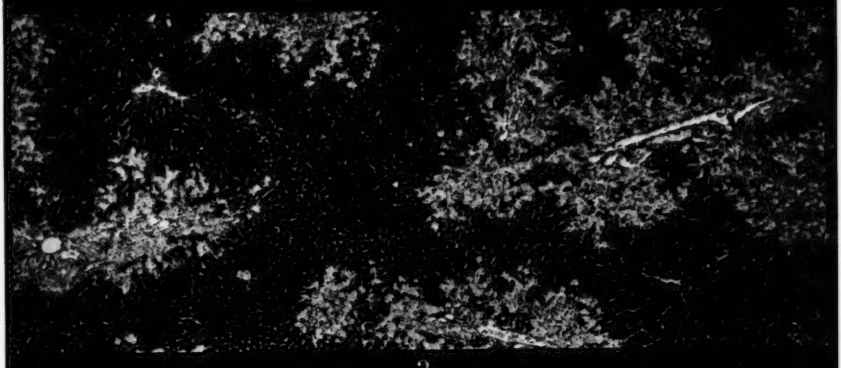

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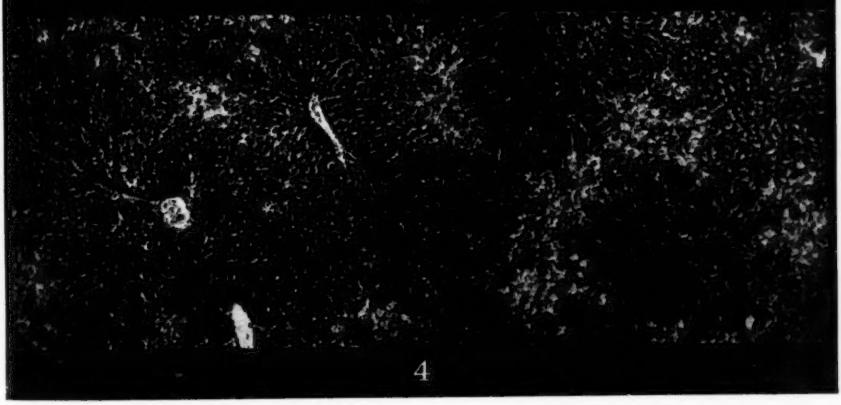

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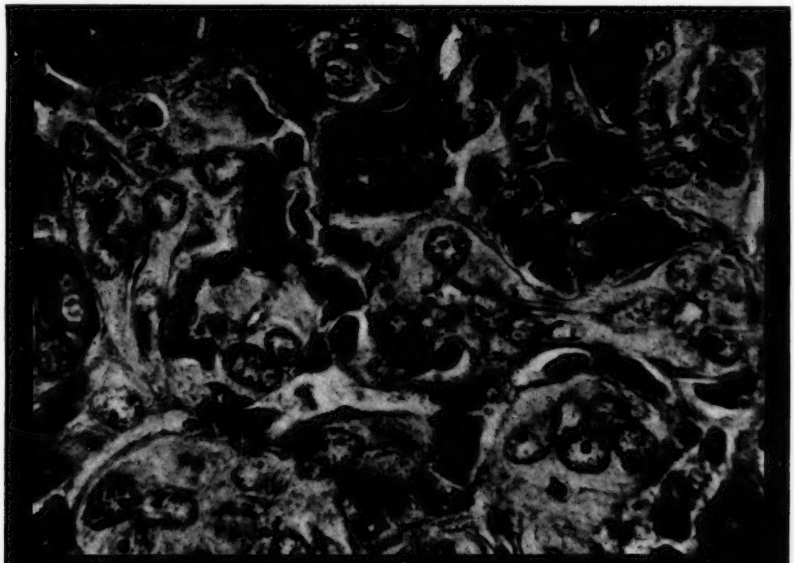

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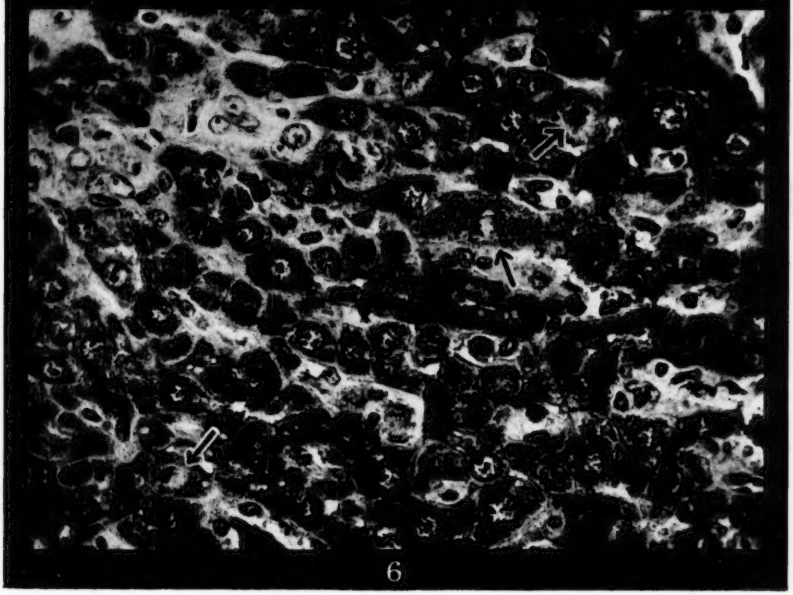

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marked ascites was the complete absence of bile duct or parenchymal proliferation. These were accordingly not included in the general weight tables and were also excluded from the tables of ratios of weight of anterior lobes to total liver weight.

Other deaths.—Twenty-one animals died as a result of operative trauma, haemorrhage and venous obstruction of residual lobes. These complications occurred usually when partial hepatectomy was attempted after long periods of bile duct obstruction. Two animals died from pneumonia.

Rats which had recovered from jaundice.—Five rats were jaundiced for periods of 15–20 days postoperatively, after which time the jaundice disappeared following spontaneous recanalisation of the common bile duct. They were killed 6–59 days later. In all cases the livers were not significantly heavier than those found in control unoperated rats. Histological examination showed almost complete regression of the proliferated bile ducts. In 2 cases there appeared to be fibrous tissue replacement of bile ducts.

DISCUSSION

One of the difficulties encountered in the interpretation of results in this series of experiments was the problem of differentiating regeneration of parenchymal cells from proliferation of smaller bile ducts following ligation of the common duct. Since both processes contribute to the increase in weight of the liver, their separation is essential to avoid confusion.

Higgins and Anderson (1932) carried out combined bile duct ligation and partial hepatectomy in rats and found that at comparable times the residual lobes weighed less than livers with biliary obstruction only. They did not, however, compare the weights of the corresponding posterior lobes in the two series. Several of their experimental animals in which both operations were performed, recovered from jaundice 21 days postoperatively. A week later the liver remnants in these cases weighed less than the control duct-obstructed livers. From these findings the authors concluded that the regenerative capacity was reduced by the procedure of bile duct ligation. The findings in the present investigation do not confirm this view and weight increase after partial hepatectomy is clearly demonstrated when the posterior lobes in both series are compared. After recanalisation and establishment of normal bile flow, the livers decrease in weight owing probably to regression of bile ducts (Stewart and Cantarow, 1935). The liver weight in non-jaundiced animals does not therefore reflect the maximum weights reached when the animals were jaundiced. After bile duct ligation and partial hepatectomy are carried out at the same operation, the residual lobes increase in weight and it seems clear that part of this increase represents the normal reaction to biliary obstruction and part represents re-creation of parenchymal tissue following partial hepatectomy. The capacity for parenchymal regeneration does not appear to be reduced when bile duct ligation and partial hepatectomy are carried out at the same operation.

Probably the best-known work so far published on the subject is by Cameron (1935), who transplanted pieces of liver into the omentum after varying periods of biliary obstruction. He observed diminished ability of parenchymal cells to grow in these grafts. From these results he concluded that this reflected a decreased capability of the liver cells to regenerate in these circumstances. Experimental bile duct obstruction in rats is thought to inhibit selective functions

of the liver cell and spare closely related processes (*e.g.*, inhibition of succinic dehydrogenase and sparing of other enzymes in the isocitric acid cycle (Edlund)). Similarly, the factors associated with survival of cells in transplanted liver may not be identical with those controlling regeneration *in situ*.

Edlund and Holmgren (1945) found hepatic parenchymal mitoses in obstructive jaundice of short duration, but fewer or none 7 days after bile duct ligation. From this they concluded that there was decreased regeneration (Edlund). In the experiments recorded in this paper, mitotic figures were found soon after bile duct ligation and were found as late as 20 days post-operatively. There is no evidence, in any case, that absence of mitotic figures indicates an absence of regenerative capacity of liver cells. In fact, 4 or 5 days after partial hepatectomy in control animals, when the liver residuum is clearly still increasing in mass, many sections may be examined which show few or no mitoses (Brues *et al.*, 1936 ; Fishback, 1929).

Cantarow and Stewart (1936) ligated the bile ducts of cats and then gave repeated intravenous injections of sodium glycocholate. They noted increased numbers of mitotic figures as a result of this treatment. They postulated that regeneration was stimulated in biliary obstruction only if bile salts were administered, and inferred that regeneration was inhibited in the absence of bile salts. Rachmilewitz, Rosin and Doljanski (1950) listed a number of tissue extracts and extrinsic substances which induce mitoses in an otherwise normal liver. In the light of this information, Cantarow and Stewart's findings may possibly be interpreted to indicate that in cats, when the correct stimulus is applied, mitoses may be induced even in the presence of bile duct obstruction.

The experiments reported in this paper were designed to test the regenerative capacity of the rat's liver in the early as well as the late stages of biliary obstruction. The findings indicate that growth of liver parenchymal cells is not inhibited or diminished by bile duct obstruction, provided that regeneration is stimulated by partial hepatectomy. It is of interest that bile duct ligation may inhibit certain functions of the parenchymal cells, but the capacity to proliferate in order to maintain a minimal mass of functioning liver tissue *in situ*, is not affected by this procedure.

One must be cautious in applying experimental findings to the elucidation of problems in human pathology. A striking difference between the liver in Laennec's cirrhosis and that in long-standing total obstructive jaundice is the frequent absence of regeneration nodules in the latter. If the reactions in man and the rat are comparable, however, it seems reasonable to suspect that there is no large-scale destruction of liver cells in bile duct obstruction and therefore without that stimulus no resultant regeneration of the surviving tissue. There is no evidence that this difference reflects a diminished regenerative capacity of liver cells in biliary obstruction.

SUMMARY.

Regeneration of liver tissue after partial hepatectomy was compared in normal rats and in rats after varying periods of bile duct obstruction.

Wet and dry weights of the residual liver tissue were measured and in selected cases, the ratio of parenchymal tissue to bile duct tissue was estimated using a slide projection method.

Regeneration of parenchymal cells did not appear to be diminished when bile duct ligation and partial hepatectomy were carried out at the same operation, nor when partial hepatectomy was performed 18–21 days after bile duct ligation.

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STUDIES ON THE ANTIGENIC STRUCTURE OF HERPES SIMPLEX VIRUS.

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THE serological diagnosis of herpes simplex infections has in the past depended to a great extent on neutralisation tests (Zinsser and Tang, 1929; Weyer, 1932; Hudson, Cook and Adair, 1936; Burnet and Lush, 1939). Complement fixation methods have also been used but the rabbit brain antigens for these tests, whilst giving satisfactory results in some hands (Kraus and Takaki, 1925, 1926) failed to do so in others (Tang and Castaneda, 1929). Bedson and Bland (1929) obtained specific complement fixation using herpetic guinea-pig pad suspensions and this method was also used by Brain (1932) with success. More recently Hayward (1949, 1950) and Dudgeon (1950) using egg-grown virus have obtained satisfactory results by complement fixation with a technique which is less time-consuming and expensive of materials than neutralisation tests for clinical serological diagnosis.

Allergic skin reactions in animals immunised with herpes simplex virus were noticed by Bedson (1931) and have since been used by Jawetz, Coleman and Allende (1951) for the diagnosis of herpes in human subjects. The latter workers further noted that the skin-reactive properties of the virus were a function of a heat-stable soluble antigen. Hayward (1949) observed that specific complement fixation depended on a heat-labile soluble antigen which could be partially separated from the virus particle. The present paper reports observations on the relationship of these antigens to complement fixation, skin reactivity and neutralisation.

METHODS.

Virus strains.—The virus used throughout this study was the "Nash" strain of herpes simplex (Kipping and Downie, 1948). The guinea-pig adapted line had been maintained in the guinea-pig pad by 68 serial passages and the egg-adapted line by 54 serial passages on the chorio-allantoic membrane of the developing hen's egg.

Virus cultivation.—Serial passages were made in the plantar skin of the guinea-pig by intradermal inoculation. (Bedson, Maitland and Burbery, 1927; Bedson and Crawford, 1927). Infection was allowed to proceed for three days, after which the guinea-pig was killed. The pads were then weighed and cut up with scissors as finely as possible. They were ground with sand in a mortar, made into a 20 per cent suspension with 10 per cent guinea-pig serum saline, and stored in a refrigerator at 4°.

Egg-cultured material was grown on the chorio-allantoic membrane by the method of Beveridge and Burnet (1946). Ten-day old eggs were used and infection allowed to proceed for 72 hr. after which the membranes were harvested. Membranes from each batch of three

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eggs were placed in a 5 ml. quantity of 10 per cent guinea-pig serum saline and were placed at 4° overnight. The following day the membranes were ground up in a TenBroeck tube in the guinea-pig serum saline mixture in which they had been standing.

Preparation of antigens.—Guinea-pig cultivated virus and egg-grown material were treated similarly. The ground suspensions were first clarified in a horizontal centrifuge at 2000 r.p.m. for 10 min. to remove gross particulate material. The supernatant was removed and this crude material was used as antigen both for immunisation and in various complement fixation and skin tests.

Further separation of the antigen was carried out by spinning the clarified product at 9000 r.p.m. in an angle centrifuge for 3 hr. The top fatty layer was discarded and the clear supernatant fluid was separated from the particulate deposit which was then re-suspended to its original volume by grinding in a TenBroeck tube with 10 per cent guinea-pig serum saline. This washing was repeated twice. The washed re-suspended deposit was used as particulate antigen and the clear supernatant fluid from the first high speed spinning as soluble antigen.

Preparation of sera.—Hyperimmune sera were prepared in the guinea-pig after first establishing infection by intradermal pad inoculation. After 14 days immunisation was continued by weekly intraperitoneal injections of 0·5 ml. clarified virus suspension. Immunisation was continued until the serum had a titre of at least 1/64 when tested by complement fixation. This took about 8 weeks.

Complement fixation technique.—Sera were inactivated at 56° for 30 min. A unit volume of 0·1 ml. diluted serum was used for each tube and an equal volume of 2 M.H.D. of diluted guinea-pig complement from a batch of pooled serum stored on dry ice was added, followed by 0·1 ml. antigen. The total volume in each tube was then made up to 0·8 ml. with saline. Fixation was carried out at 4° overnight and racks were left for 30 min. at room temperature before adding 0·2 ml. sensitised sheep cell system. This consisted of 5 M.H.D. haemolytic amboceptor in a 5 per cent sheep cell suspension. The tubes were then placed in a 37° water-bath for 30 min. before reading. A further reading was also made after 24 hr. Results were recorded as ++++ for complete fixation and a lesser number of + signs according to the amount of haemolysis.

Preliminary tests using different quantities of complement from 1·5 to 2·5 M.H.D. and varying times of fixation indicated that overnight fixation at 4° with 2 M.H.D. of complement was the most satisfactory method.

Neutralisation tests.—These were done in the shaved skin of the albino guinea-pig. Serum virus mixtures were allowed to stand for 30 min. at room temperature and 0·2 ml. was then injected slowly into the skin with a fine needle. Titrations were made using varying concentrations of both serum and virus. Areas of induration were measured in cm. after 24, 48 and 72 hr.

RESULTS.

Comparison of complement-fixing activity of soluble and particulate antigens.

Evidence exists that the complement-fixing activity of herpes antigens is chiefly due to a " soluble " specific substance which may be separated from the virus particle by centrifugation for a period of 1 hr. at 8000 r.p.m. (Hayward, 1949). In order to confirm this, soluble antigen was prepared from infected chorio-allantoic membranes by the method already described. The particulate antigen was washed, ground and re-suspended in guinea-pig serum saline five times in all in order to achieve as complete separation from the soluble antigen as possible. These antigens were tested by complement fixation.

Table I shows that the washed particulate antigen possesses only slight complement-fixing activity whereas the soluble antigen shows a degree of activity comparable with that of the original whole suspension. Tests were also done using fewer changes of serum saline for washing. These showed markedly more complement-fixing activity in the deposit.

TABLE I.—*Titration of Complement Fixing Activity of Separate Antigen Fractions.*

Sera.		Antigens. Crude antigen. 1/2	1/4	1/8	Soluble antigen. 1/2	1/4	1/8	Particulate antigen. 1/2	1/4	1/8
H63—										
Hyperimmune serum	1/32	++++	+++	+	++++	+++	+	±	±	−
		++++	+++	+	++++	+++	−	−	−	−
Normal guinea-pig serum	1/16	−	−	−	−	−	−	∓	∓	−
		−	−	−	−	−	−	−	−	−
Saline	..	−	−	−	−	−	−	−	−	−
		−	−	−	−	−	−	−	−	−

The first and second readings for each tube are shown.

Stability of the soluble antigen.

Hayward (1949) demonstrated that the soluble antigen is very heat-labile as indicated by complement fixation. Soluble antigen was heated at 56° for 30 min. and used with similarly treated particulate antigen for complement fixation.

Table II shows that heating at 56° for 30 min. completely destroys the complement-fixing activity of the antigen. It also appears to make the particulate antigen rather more anti-complementary.

TABLE II.—*Effect of Heat on Separate Antigen Fractions.*

Sera.		Antigens. Soluble. Unheated. 1/2	1/4	1/8	Soluble. Heated. 1/2	1/4	1/8	Particulate. Unheated. 1/2	1/4	1/8	Particulate. Heated. 1/2	1/4	1/8
H63—													
Hyper-immune serum	1/32	++++	+++	+	−	−	−	±	±	−	+	±	±
		++++	+++	+	−	−	−	−	−	−	+	±	±
Normal guinea-pig serum	1/16	−	−	−	−	−	−	∓	∓	−	±	±	±
		−	−	−	−	−	−	−	−	−	−	−	−
Saline	..	−	−	−	−	−	−	−	−	−	±	−	−
		−	−	−	−	−	−	−	−	−	−	−	−

It was noted that after the separation of the soluble antigen from the particulate virus the complement-fixing activity of the former declined very much more rapidly than in the unseparated membrane suspensions. The addition of sodium azide to the separated soluble antigen effectively preserved its activity.

Table III shows the effect of storage at 4° for 8 weeks on the complement-fixing activity of the crude suspension and of the soluble antigen with and without the addition of 0·3 per cent sodium azide. It can be seen that azide preserves the activity of the soluble antigen, but has little apparent effect on the unseparated suspension which retains its activity well even when untreated. The action of 0·2 per cent formalin and of thioglycollate were also tested; both preserved the

TABLE III.—*Stability of Soluble Antigen as Tested by Complement Fixation.*

Sera.		Antigens (after 8 weeks storage at 4° C.). Soluble. 1/2	Soluble. 1/4	Soluble + 0·3 per cent azide. 1/2	Soluble + 0·3 per cent azide. 1/4	Unseparated antigen. 1/2	Unseparated antigen. 1/4	Unseparated antigen + 0·3 per cent azide. 1/2	Unseparated antigen + 0·3 per cent azide. 1/4	Normal C.A.M. 1/4	Saline.
11/52 Hyperimmune guinea-pig serum	1/32	+	∓	++++	++	++++	++	++++	++	−	−
		∓	−	++++	+	+++	+	+++	+	−	−
Normal guinea-pig serum	1/16	−	−	−	−	−	−	−	−	−	−
		−	−	−	−	−	−	−	−	−	−
Saline	..	−	−	−	−	−	−	−	−	−	−
		−	−	−	−	−	−	−	−	−	−

complement-fixing activity to some extent but they were less effective than sodium azide.

Acids and alkalis were found to destroy the complement-fixing activity of the soluble antigen. This was tested by standing the antigen at pH 5·4 and 8·0 overnight at 4°. The solutions were then neutralised to pH 7·0 and all trace of complement-fixing activity could then be shown to have disappeared.

Comparison of skin reactivity of soluble and particulate antigens in normal and hyperimmune animals.

Suspensions of herpes simplex virus will infect the skin of the guinea-pig and the titre of infectivity of the virus may be measured in this way (Bedson and Crawford, 1927). The immunity of the guinea-pig may also be measured by this means and dermal reactivity has been used in the human subject in order to demonstrate hypersensitivity to the virus antigens (Jawetz, Coleman and Allende, 1951).

Experiments were therefore carried out in order to show the effect of intradermal inoculation of the separated antigens in both normal and hyperimmune animals and the effect of heat on these antigens in order that dermal reactivity might be correlated with complement-fixing activity.

Twenty per cent guinea-pig pad suspensions were used throughout these dermal tests in order to obviate difficulties which might arise from non-specific reactions to egg-grown material. The complement-fixing activities of the guinea-pig pad fractions were tested in parallel with the skin tests.

Tenfold serial dilutions of the purified antigens were made in guinea-pig serum saline. Heated antigens were placed in a water-bath at 56° for 30 min. The titration was done in the shaved skin of the guinea-pig using 0·2 ml. amounts. Readings were made after 24, 48 and 72 hrs. The hyperimmune animals were those immunised for the production of sera as already described.

Fig. 1 shows that in the normal guinea-pig the soluble, heated soluble and heated particulate antigens evoked a negligible reaction. The unheated particulate antigen produced infection with lesions that showed increasing induration up to the third day and thereafter faded away. These lesions were accompanied by only minimal erythema which was hardly perceptible on the first day, but

tended to increase on the following days. At no time did they reach a diameter exceeding the area of induration by more than 0·5 cm.

In contrast the hyperimmune animals showed well marked areas of induration which appeared within a few hours with both the soluble and heated soluble antigen and a rather lesser reaction to the heated particulate antigen. These areas were surrounded by an intense erythema often twice or three times the area of induration. This erythema faded rapidly and had in all cases disappeared after 48 hours. The areas of induration also subsided rapidly. On the other hand the washed particulate antigen in the hyperimmune animal showed its maximum area of induration after 24 hours and unlike the normal animal either became smaller in area or remained about the same size.

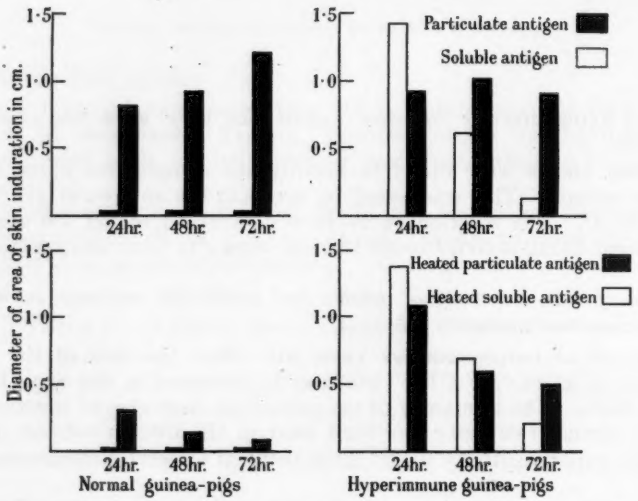

FIG. 1.—Skin reactivity to heated and unheated soluble and particulate antigens in normal and hyperimmune guinea-pigs.

There is therefore a clearly marked difference between these two groups of animals; the hyperimmune on the one hand, which showed marked dermal sensitivity to heated and unheated soluble antigens with partial resistance to the particulate virus, and the normal animals on the other which showed no reactivity to the soluble antigen and a progressive infective process when inoculated with the unheated washed virus.

Although the soluble fraction was, in these experiments, unaffected by heat, this treatment had been found to destroy all complement-fixing activity (Table II).

Preparation of antisera to soluble and particulate antigens.

The soluble and particulate antigens were prepared freshly for each inoculation and were treated with 0·2 per cent neutral formalin immediately before use. Injections of 0·5 ml. were made weekly into the peritoneum of guinea-pigs for a period of 9 weeks and in the case of the soluble antigen this was continued for a

further 5 weeks. At the end of this time animals were bled by cardiac puncture 6 days after the last inoculation.

Sera were tested by complement fixation and Table IV shows the results using two such sera, 37/52 and 38/52, prepared by immunisation of guinea-pigs with particulate antigen. It may be seen that they have very little complement-fixing activity as compared with hyperimmune serum (Table IV).

TABLE IV.—*Titration of Complement-fixing Activity of Sera Prepared by Immunising Guinea-pigs with Washed Virus.*

Antigens.	Sera prepared against— Whole virus 11/52. 1/32	1/64	1/128	Particulate antigen 37/52. 1/4	1/8	1/16	Particulate antigen 38/52. 1/4	1/8	1/16	Normal guniea-pig serum. 1/16	Saline.
H62—											
C.A.M. 1/4	++++	+++	+++	∓	±	∓	±	±	−	−	−
antigen	+++	+++	+++	−	+	−	−	−	−	−	−
Normal 1/4	−	−	−	−	−	−	−	−	−	−	−
chorio-allantoic membrane	−	−	−	−	−	−	−	−	−	−	−
Saline	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−

Table V shows that the neutralising activity of the sera 37/52 and 38/52 are comparable with that of the hyperimmune serum 11/52. Thus sera prepared by

TABLE V.—*Neutralising Activity of Sera Prepared from Guinea-pig Immunised with Washed Virus.*

			Readings at 48 hr. Virus dilutions. Undiluted.	10^{-1}	10^{-2}	10^{-3}	10^{-4}
G.P. 83/52	Normal guinea-pig serum	1/30	+++	++	+	+	−
	11/52 Hyperimmune guinea-pig serum (living virus)	„	++	+	−	−	−
	37/52 Test serum (washed virus)	„	++	+	−	−	−
G.P. 84/52	Normal guinea-pig serum	„	+++	++	+	+	−
	11/52 Hyperimmune guinea-pig serum	„	++	++	−	−	−
	38/52 Test serum	„	++	+	−	−	−

+ = an area of erythema approximately 0·5 cm. in diameter.

immunising animals with particulate antigen possess full neutralising activity but little or no complement-fixing antibody.

Sera prepared by inoculation of soluble antigen failed to produce more than a trace of complement-fixing antibody and possessed no neutralising activity. This finding is discussed below.

The skin reactivity of animals immunised with soluble and particulate antigens.

Animals which had received immunisation with the separated antigens for the purpose of preparing antisera were tested for their dermal reactivity. Fig. 2 shows that the animals immunised with soluble antigen were sensitive to this fraction but not immune to infection with washed virus. Those that had received killed particulate antigen showed some immunity as compared with the normal control, but no evidence of dermal reactivity.

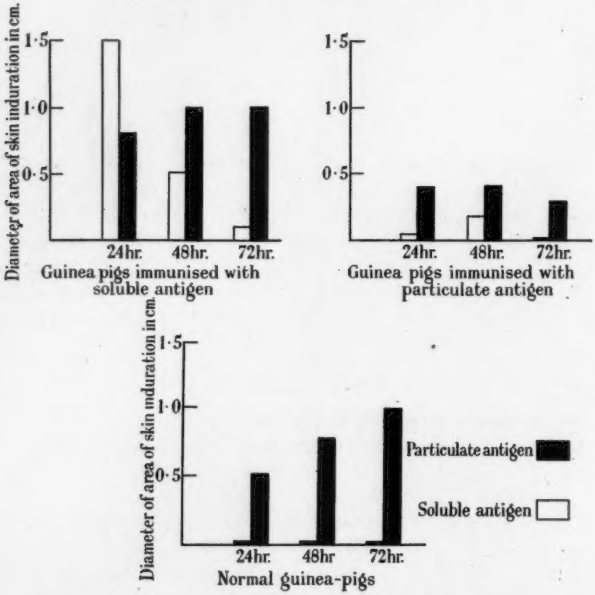

FIG. 2.—Skin reactivities to soluble and particulate antigens of guinea-pigs immunised with separate antigen fractions.

Absorption from hyperimmune sera of antibodies to the soluble and particulate antigens.

In order to demonstrate a qualitative difference in the antigenicity of the soluble and particulate fractions, attempts were made to absorb the homologous antibody for one or other fraction from a hyperimmune serum.

The particulate antigen presented no difficulty in this respect, as the antibody could be directly absorbed on the virus particle and the complex formed removed by centrifuging. For this purpose undiluted particulate antigen was used in the presence of a hyperimmune serum diluted 16-fold. The mixture was placed overnight at 4° and the particulate matter then separated by centrifuging for 3 hr. at 9000 r.p.m. in an angle centrifuge. The supernatant sera were inactivated at 56° for 30 min. and were then tested by complement fixation (Table VI). The absorbed hyperimmune serum showed complete loss of neutralising activity when tested in the guinea-pig skin.

TABLE VI.—*Titration of Hyperimmune Sera Absorbed with Particulate Antigen.*

Antigens	Sera. Hyperimmune serum.	Hyperimmune serum adsorbed with particulate antigen.			Normal guinea-pig serum.	Saline.
	1/32	1/16	1/32	1/64	1/16	
Herpetic chorio-allantoic membrane antigen 1/4	++++	++++	++++	++++	−	−
	+++	+++	+++	+++	−	−
Normal chorio-allantoic membrane 1/4	−	−	−	−	−	−
	−	−	−	−	−	−
Saline	−	−	−	−	−	−
	−	−	−	−	−	−

Efforts to obtain a precipitin reaction between the soluble antigen and the hyperimmune sera failed. It was not therefore possible to remove directly any antibody reacting with this antigen. For this reason attempts were made to attach the soluble component to some inert particle before carrying out absorption. Human red cells, red cell stroma, killed *Bact. coli* suspensions and collodion particles at pH 5·8, 7·0 and 8·0 with and without the presence of guinea-pig complement were used. In no instance was the antigen successfully attached to the particle.

DISCUSSION.

It has been shown that suspensions of herpes virus can be separated by centrifuging into a deposit of infective material and a soluble portion which possesses antigenic properties. The soluble component is both able to fix complement and also to evoke skin sensitivity reactions in the hyperimmune animal. It is not clear if these two properties represent different manifestations of the same antigen or whether two distinct antigens are responsible. Jawetz, Coleman and Allende (1951) demonstrated dermal reactivity in the human herpetic subject to heated herpes antigens. This has been confirmed in the guinea-pig and it has also been shown that the degree of heat (56° for 30 min.) which destroys the complement-fixing activity of the antigen has little appreciable effect on the dermal reaction. This would seem to suggest two separate antigens, one heat-labile and complement-fixing, the other heat-stable and dermal-reactive. On the other hand the possibility of this being a simple quantitative or physical difference is not excluded by these experiments.

The complement-fixing properties of the soluble antigen are destroyed slowly even at 4° once it has been separated from the original tissue suspensions. This destruction does not occur in the presence of the tissue and is considerably slowed by the addition of sodium azide or less markedly by thioglycollate or formalin. The reason for this remains obscure, but it seems possible that the destruction is enzymic in nature.

The heated particulate antigen has also been shown to give rise to a moderate skin reaction in the sensitised animal. Some of this may be tissue reaction to inoculation of particulate foreign material but it seems likely that it may be due mainly to the release of some of the relatively heat-stable soluble antigen from the virus particle.

The sera prepared by immunising guinea-pigs with killed particulate antigen contained neutralising antibodies to the virus particle to a titre comparable with that produced by infection. These sera, as might be expected, have very little complement-fixing activity. The guinea-pigs in which this type of serum was prepared showed no skin sensitivity to the soluble antigen, but they were at least partially immune to infection in the skin. Those which had received repeated injections of the soluble antigen failed to produce complement-fixing antibody possibly because the soluble material was removed from the tissues too quickly for effective stimulation. However, these animals showed marked allergic responses to the soluble antigen either heated or unheated, but were not resistant to infection.

It was found possible to absorb the neutralising antibody from the hyperimmune sera because the washed virus particles which were used for this purpose could be centrifuged from the mixture. Attempts to attach the soluble antigen to various types of particle as a preliminary to the absorption of the serum did not succeed. The reason for this is not clear.

The soluble antigen may arise as a metabolic product of the virus particle or as a breakdown product from its disintegration. No evidence to support either possibility has come to light in the present work. The suggestion put forward by Jawetz, Coleman and Allende (1951) that it is a precursor of the virus particle would appear to be difficult of proof and further work is clearly required.

SUMMARY.

The antigens of herpes simplex virus (" Nash " strain) have been separated into soluble and particulate components.

The soluble component both possesses complement-fixing properties and stimulates dermal reactivity in the guinea-pig. The complement-fixing properties are rapidly destroyed by heat, but skin reactivity is unaffected.

Antisera to the particulate antigen have been prepared by immunisation of animals with washed virus. These sera possess neutralising properties but lack complement-fixing power.

Antisera to the soluble antigen have been prepared by absorbing anti-herpes sera from hyperimmune guinea-pigs with washed virus particles. Such absorbed sera retain their complement-fixing power but lose their neutralising properties.

The origin of the soluble antigen is discussed.

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OBSERVATIONS ON THE POLIOMYELITIS COMPLEMENT-FIXATION TEST.

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THE failure of numerous attempts to produce a satisfactory poliomyelitis virus complement-fixing antigen has been ascribed to the low virus content of the brain and cord of susceptible adult animals, to the difficulty of concentrating the antigenic material and to the character of the adult tissues themselves. Loring, Raffel and Anderson (1947) gave a preliminary account of an antigen made from ultracentrifuged cotton-rat nervous tissue. Theirs was the only promising report until Casals and Olitsky (1950) described an acetone-ether extracted antigen prepared from suckling mouse brain (Casals, Olitsky and Anslow, 1951*a*). By serial intracerebral passage of the MEF_1 strain of type 2 poliomyelitis virus in 1- to 4-day-old mouselings they obtained an "adapted" strain with an LD_{50} of 10^{-4} to 10^{-5} as tested by intracerebral inoculation of 0·03 ml. into 3- to 4-week-old mice (Casals, Olitsky and Anslow, 1951*b*). After about the 75th suckling mouse passage, the titre in adult mice had reached $10^{-5.5}$ to 10^{-6} and the titre of antigen preparations had also increased. The virtue of mouseling brain as antigen appears to depend both on its high content of virus and on its physical and chemical constitution (Casals, 1951 ; Casals and Olitsky, 1951 ; Casals, Olitsky and Sabin, 1952).

The antigen of Casals and Olitsky is made from infected mouseling brains extracted with acetone and ether, the residue being re-suspended in physiological saline. Mouse brain antigens in general may give non-specific reactions with WR-positive and some other human sera (Casals, 1947) ; the extraction with acetone and ether (Casals, 1949) or benzene (DeBoer and Cox, 1947) is intended to eliminate them.

The experiments described in this paper were made with a simpler mouseling brain antigen, not extracted with fat solvents. Their purpose has been to confirm the specificity of the complement-fixation (CF) reaction between mouseling-"adapted" MEF_1 antigen and homologous hyperimmune and convalescent animal sera, and to study the use with this system of Fulton and Dumbell's (1949) CF technique, which has certain advantages. These studies are preliminary to work with human sera, a few of which have been tested.

In view of a projected investigation of tissue culture antigens, and the possibility that the danger of infection in the laboratory might be greater with these than with the mouseling-"adapted" virus, an attempt was made to produce a non-infective CF antigen. Some experiments on inactivation of the mouseling antigen are reported in this paper.

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MATERIALS AND METHODS.

Antigens.

Two- to three-day-old mouselings of different strains were inoculated intracerebrally with 0·01 ml. of a 10 per cent suspension in distilled water of mouseling brains infected with "adapted" MEF_1 virus. After 36 to 48 hr., by which time 75 to 100 per cent showed paralysis of one or more limbs, the mouselings were anaesthetized with ether, exsanguinated and the brains placed in screw-capped bottles on dry ice until a sufficient number, 40 or more, had been collected to make a batch of antigen. The brains were then weighed and allowed to thaw at room temperature, when they rapidly became diffluent and could easily be emulsified in veronal-saline by being forced in and out of a syringe carrying a well-fitting, sawn-off needle of internal diameter 0·5 to 1 mm. (as suggested by Dr. J. L. Melnick, personal communication). The antigen was usually made up as a 1 in 4 suspension (*i.e.*, 1 part by wet wt. of brain plus 3 parts by vol. of diluent). The apparently homogeneous milky suspension was sealed in glass ampoules and frozen and thawed three times at about −70° and 37°. Finally, the ampoules were kept on dry ice for several hours, usually overnight, and the contents then thawed and spun at 20,000 ***g*** for 1 hr. The faintly opalescent supernatant was used as antigen ("M" antigen). It was stored in sealed glass ampoules on dry ice. Six mouselings yielded about 1 g. of brain tissue or 2·5 to 3 ml. of 1/4 antigen.

This method of preparing antigen is based on that of Casals and Palacios (1941). Different samples of antigen were of like opalescence, whether made from normal or infected brains. If further flocculation occurred when the antigen was thawed for use, clarification by centrifugation did not reduce the titre, but this treatment was seldom necessary.

Antigens were also prepared from normal 4–5-day-old mice ("N" antigen) and from mice of similar age infected with the FA strain (Theiler and Gard, 1940) of mouse encephalomyelitis virus ("FA" antigen).

Using the Lansing (Armstrong) strain of type 2 poliomyelitis virus in its first passage in baby cotton-rats, Lahelle (1951) obtained an effective CF antigen. An attempt was made in this laboratory to produce a Lansing antigen by direct intracerebral inoculation of baby mice but without success.

Sometimes two and occasionally three successive litters from the same mother mouse were used to make M antigen. There was no obvious difference in the susceptibility of these litters to the virus, nor in their suitability for antigen preparation.

Sera.

Normal sera of: adult mouse; cotton-rat; hamster; guinea-pig; monkey (individual pre-inoculation sera and a pool of 8 normal sera); human (sera of 1–2-year-old children with no history of poliomyelitis, made available by Dr. J. A. Dudgeon of the Hospital for Sick Children, Great Ormond Street).

"Control" sera from: mice inoculated intraperitoneally with normal adult mouse brain; cotton-rats inoculated with adult cotton-rat brain; hamsters and guinea-pigs inoculated with adult mouse brain.

Hyperimmune sera: (*a*) against Lansing virus, from mice and cotton-rats hyperimmunized with homologous infected brain;

(*b*) against "adapted" MEF_1 virus, from mice, hamsters and guinea-pigs hyperimmunized with infected mouse brain;

(*c*) against the FA strain of Theiler's virus, from mice and cotton-rats hyperimmunized with homologous infected brain;

(*d*) against poliomyelitis virus of types 1, 2 and 3 (Brunhilde, Lansing and Leon strains), from rhesus monkeys hyperimmunized with infected monkey cord mixed with adjuvant.

Hyperimmune and "control" sera were prepared in mice, cotton-rats, hamsters and guinea-pigs by giving 5 twice-weekly intraperitoneal injections of a 10 per cent aqueous suspension of normal or infected brain, inocula ranging from 0·25 ml. to 1·0 ml. according to the animal's size. Animals were anaesthetized with ether and bled from the axilla 2 weeks after the last injection, without preliminary test bleedings.

Monkey hyperimmune sera were received from Dr. Alan Goffe of the Virus Reference Laboratory, Colindale. The course had consisted of 3 intramuscular injections of 4 ml. of an emulsion containing equal parts of 20 per cent suspension of infected monkey cord and an

adjuvant mixture of Bayol F, 9 parts and Arlacel A, 1 part, (Salk, Lewis, Bennett and Youngner, 1951). Intervals between injections were 2 and 3–6 weeks. Human γ-globulin, 750 mg., was given with the first dose to lessen the danger of paralysis. Blood was taken before the first injection and at different intervals thereafter.

Convalescent sera from monkeys were received from Dr. F. O. MacCallum of the Virus Reference Laboratory, Colindale. The monkeys had recovered, after intracerebral inoculation, from paralytic Lansing infections or had been inoculated with samples of sewage or faeces and had shown histological lesions in the CNS characteristic of poliomyelitis, with or without paralysis.

A pool of convalescent serum was obtained from 12 hamsters which had developed paralysis of one or more limbs after intracerebral inoculation of " adapted " MEF_1 virus and had recovered. They had been part of a group of animals used in an attempt to titrate this virus in hamsters. The serum was taken 6 weeks after inoculation.

Specimens of " acute phase " and " convalescent phase " sera, from patients diagnosed clinically as cases of paralytic poliomyelitis, were received from Dr. F. O. MacCallum and from Dr. C. L. Greenbury of the N.W. Regional Group Laboratory. Sera from healthy adults, as well as WR-positive specimens, were provided by Dr. R. A. Shooter of St. Bartholomew's Hospital.

All rodent sera were kept as pools of serum from at least 12 animals. Hyperimmune mouse sera and some others were kept at − 70° and the rest at − 20°. At first all sera were inactivated at 56° for 30 min. immediately before use ; later, all human and monkey sera and some mouse and hamster sera were inactivated at 60° for 20 min. according to the practice of Casals. Sera were diluted 1 in 4 before inactivation except those used and inactivated at 1 in 2.

Diluent.

0·85 per cent sodium chloride in glass-distilled water containing calcium and magnesium ions and buffered with veronal at pH 7·2 (Mayer, Osler, Bier and Heidelberger, 1946).

Complement.

Fresh guinea-pig serum stored in sealed glass ampoules on dry ice.

Haemolytic system.

Washed sheep red cells 0·2 per cent, standardized photometrically and sensitized with an equal volume of 1 in 400 Burroughs Wellcome & Co. haemolysin. This represents an excess of antibodies sufficient to permit agglutination of any unlysed cells and so facilitate the reading of results (see p. [305] : *Addition of sensitized cells*). This concentration of haemolysin is equivalent to about sixteen 50 per cent haemolytic doses.

CONDUCT OF THE TEST.

Reagents were titrated on perspex plates according to the technique of Fulton and Dumbell (1949). The test is a 5-volume test, with a unit vol. of 0·02 ml. The first phase proceeds for 18 hr. at 4°, the second for 2 hr. at 37°. Each plate is put away in the cold box as soon as the drops of serum, complement and antigen have been distributed upon it, in order to prevent evaporation. The apparatus and the methods of titrating, reading and calculating results were as described in the original paper except for the amplifications and points of difference noted below. These are given at some length, as likely to assist anyone using the method with poliomyelitis reagents. They should be read in conjunction with the original paper.

Apparatus.

The perspex plates were ruled into 10 × 10 squares, instead of 12 × 12.

Dropping pipettes were made to deliver drops of 0·02 ml. and, for the haemolytic system, of 0·04 ml. They were individually tested for accuracy by weighing an estimated 1 ml. of saline. Only these pipettes were used in the tests. Those that had held infective antigen were allowed to stand for at least 30 min. in 2N HCl before being cleaned. This makes the

virus non-infective as judged by intracerebral inoculation of mice. After use all perspex plates were left for at least 30 min. in 2N HCl and then washed in tap water and distilled water.

Titration of sera.

The fixation of complement may be explored in one, two and three "dimensions." The "three-dimensional" test was generally used for the examination of a new batch of antigen (see below, p. [305]: *Titration of antigen*). For the titration of sera, both "one-dimensional" or "line" and "two-dimensional" tests were used.

Line tests.—Here, only the serum concentration is varied, and complement is referred to as the dose, *i.e.*, the total number of units, initially added. The value for the unit, in

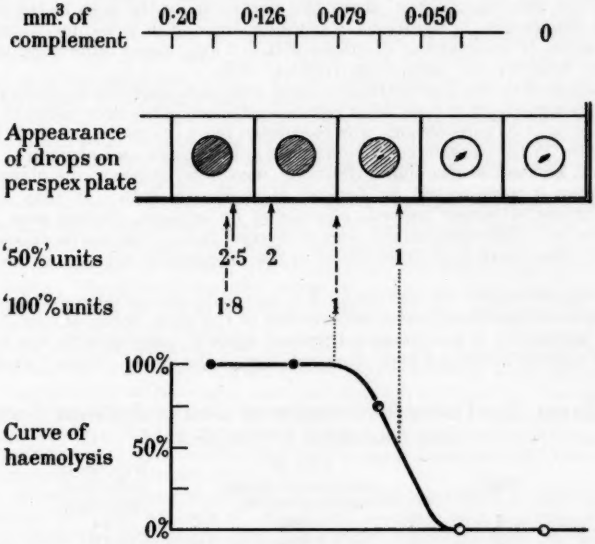

FIG. 1.—A comparison of different types of complement unit.

	"50 per cent" unit.	"100 per cent" unit.
Comparative strengths of different types of unit	U = 0·056 mm.³	U = 0·08 mm.³
	2·5 U = 0·14 mm.³	1·8 U = 0·144 mm.³
	2 U = 0·112 mm.³	

mm.³ of neat guinea-pig serum per 0·02 ml. unit volume, is judged from previous titrations of the same batch of complement (see p. [304]).

Serial dilutions of serum are tested against a single optimal concentration of antigen with a single convenient dose of complement. The modest degree of specific fixation obtained with most poliomyelitis convalescent sera demands that the dose of complement be as small as is consistent with clarity and with unequivocal haemolysis in controls. In these tests the dose selected was 2·5 units, but if the anticomplementary effect of sera and non-specific reactions can be eliminated and if the slope of the haemolysis curve is steep enough in the presence of each antigen used, then it may be worth while to use less than 2·5 units. The haemolysis curve in Fig. 1 is steep enough for as little as 2 units to give complete haemolysis.

Two-dimensional tests.—Not only is the serum concentration varied but different levels of complement are used, *i.e.*, different amounts of neat guinea-pig serum per 0·02 ml. unit volume. The number of units which each amount represents is not known at the outset.

The value for the complement unit is learned during the test itself and results are then expressed as the number of units, or $\log_{10}$ units, fixed at each serum dilution (*vide infra*). A value of 0·5 unit, *i.e.*, 1·7 $\log_{10}$ unit, has been taken as the lowest level of significant complement fixation. Any fixation below this level is disregarded. The antigen concentration chosen is that which is optimal at this level.

Usually eight dilutions of complement are used in these tests (see below : *Titration of complement*) ; but with a few of the human sera only the five highest dilutions were used, in a restricted two-dimensional test, to save space on the plates. This modification is possible because poliomyelitis convalescent sera usually fix relatively small amounts of complement.

Titration of complement.

The unit of complement.—The unit is that amount, in mm.³, of neat guinea-pig serum which gives 50 per cent haemolysis, under the conditions of the test, in the presence of the antigen. It is convenient, in titrations of complement and in two-dimensional tests, to use a logarithmic series of complement dilutions with 0·2 $\log_{10}$ mm.³ differences in 0·02 ml. unit volume. Eight dilutions are used, from 1/20 to 1/500.

Since the antigens in the concentrations used were not anticomplementary and since the strength of the complement did not alter significantly during the time taken to set up the test (up to 2 hr.), the unit of complement was calculated from the mean value of the readings on the various plates in the control rows containing complement and antigen.

Complement, at least in the higher dilutions, may deteriorate on the plates in the course of the test unless it is protected by antigen or serum or both. It may also fall off in the higher dilutions of serum control, containing no antigen, though sera differ in their power to protect it. The complement unit computed from dilutions in diluent alone would be erratic and higher than that determined in the presence of antigen, which is always the unit used.

Choice of complement unit for line tests.—For line tests the complement dose is based on a provisional unit obtained from previous titrations of the same batch of complement and the actual unit is checked by a complement titration made in parallel with the test. The dose of complement therefore varies a little from one test to the next. Table I shows the amount

TABLE I.—*Values for Complement Unit in Different Tests with One Batch of Complement.*

Test.	Unit of complement (mean value in mm.³).	Number of individual readings.
1	0·056	24
2	0·062	12
3	0·053	6
4	0·059	20
5	0·063	4
6	0·058	8
7	0·058	16
8	0·053	15
9	0·060	12
10	0·057	12

of variation to be expected in ten different tests with one batch of complement. The value shown for each test is the mean of several readings, usually 12 to 16, made in the presence of the selected concentration of normal or infected antigen. The unit varies in this series between 0·053 and 0·063 mm.³

The frequency of individual readings observed in one test (No. 4) was as follows :

0·050 mm.³ × 3
0·056 mm.³ × 8
0·063 mm.³ × 7
0·071 mm.³ × 2

Evaluation of complement unit in two-dimensional tests.—In the two-dimensional test serial complement dilutions are used in test and controls. The unit of complement is deter-

mined during each test and exact initial strength does not matter. Fixations of as little as 0·5 unit can therefore be read with confidence. The line test could only achieve this sensitivity if the dose of complement used were 1·5 units (≡ 0·5 unit available for fixation) instead of the 2·5 units necessitated by the limitations of the test.

Different methods of reading the complement unit.—Casals, Olitsky and Anslow (1951*a*) used between 1·6 and 2·0 units, usually 1·8 units. Their unit was the least amount of complement, in an arithmetic series of dilutions, required to give 100 per cent haemolysis. The result of a representative complement titration by the plate technique is shown in Fig. 1. It may be seen that 1·8 100 per cent units and 2·5 50 per cent units are nearly the same.

Titration of antigens.

Standard concentration of antigen.—In tests to compare antigenic relationships each antigen is used at a standard concentration defined as that concentration which fixes specifically a convenient amount of complement in the presence of an excess of homologous antiserum. For example, for type 2 poliomyelitis virus (M) antigen, 0·6 $\log_{10}$ unit of complement was chosen and a 1/4 dilution of MEF_1 hyperimmune mouse serum provided a relative excess of homologous antibody. In the three-dimensional test shown in Fig. 2 the standard concentration of antigen under these conditions lay between 1/16 and 1/32.

Optimal concentration of antigen.—To detect specific antibodies in different sera an antigen is used at a concentration optimal for that purpose at the complement level selected (*cf.* Hoyle, 1945). Different preparations of M antigen used for this purpose were titrated against a standard pool of homologous mouse antiserum.

Three-dimensional tests.—Three-dimensional tests, in which the concentrations of serum, complement and antigen are varied, have been used for the examination of each new batch of antigen. They are somewhat extravagant of reagents but they provide the fullest available picture of a particular CF system. Both standard and optimal antigenic titres may be learned from them. They also reveal any zones of inhibition which may occur in excess of antigen or serum, as well as the presence and degree of any anticomplementary or procomplementary power in either reagent.

The graph of a three-dimensional test is shown in Fig. 2. The areas of fixation obtained with doubling dilutions of M antigen from 1/8 to 1/64 were 56·4, 45·1, 32·7 and 23·1 cm.^{2}; only the 1/8 concentration of N antigen gave any fixation (1·0 cm.^{2}). The maximum serum titre was taken as the highest dilution of the series to give 50 per cent or greater fixation, at the 0·18 $\log_{10}$ unit level for the purposes of the line test, and at the $\bar{1}$·7 $\log_{10}$ unit level for the two-dimensional test. This serum titre had in most cases already been attained with an antigen concentration of 1/16 or less.

In general, in testing batches of antigen, no pro-zone was found in the region of highest antigen concentration. The absence of antigen excess zones with unextracted mouse brain antigens agrees with the experience of Casals (1949), although both he and España and Hammon (1948) found antigen excess zones with antigens extracted with acetone and ether or with benzene. With the unextracted antigen, there is therefore a range of antigen concentrations over which a maximum serum titre may be obtained. A concentration in the more dilute half of the range is selected for the test in order to conserve supplies of antigen. In the example shown in Fig. 2, a 1/4 concentration of antigen was not included; it had been found with earlier batches of antigen that the serum titre was no higher than with the 1/8 concentration. It will be seen that at the 0·18 $\log_{10}$ unit level (≡ 1·5 units of specifically fixed complement or 2·5 units of total initially added complement, as used in the line test), the antigen concentrations of 1/8 and 1/16 both give a serum titre of 1/128; while at the $\bar{1}$·7 $\log_{10}$ unit line, the base-line for the two-dimensional test, the same two concentrations of antigen give a serum titre of 1/256. An antigen concentration of 1/12 to 1/16 would therefore be used in both kinds of test.

Whenever M antigen or any other infected antigen was used, a parallel test was set up with normal antigen in the same concentration.

Addition of sensitized red cells.

The formation of a clear-cut " button " of unlysed cells depends not only upon the presence of agglutinating antibodies in the anti-sheep red cell serum, but also on the concomitant action of a heat-stable factor present in various sera and in the serum of guinea-pigs in higher titre than complement. In the control drop containing only sensitized cells the characteristic button of agglutinated cells is not seen, even after gentle tapping of the plates.

Before adding the drops of the sensitized cell suspension it is helpful to allow an electric fan to play gently upon the plates for one to two minutes in order to cause the condensed film of moisture on the surface of the perspex to evaporate. Unless the perspex between the drops is dry they will tend to spread over the surface during the subsequent incubation at 37° and may even run together.

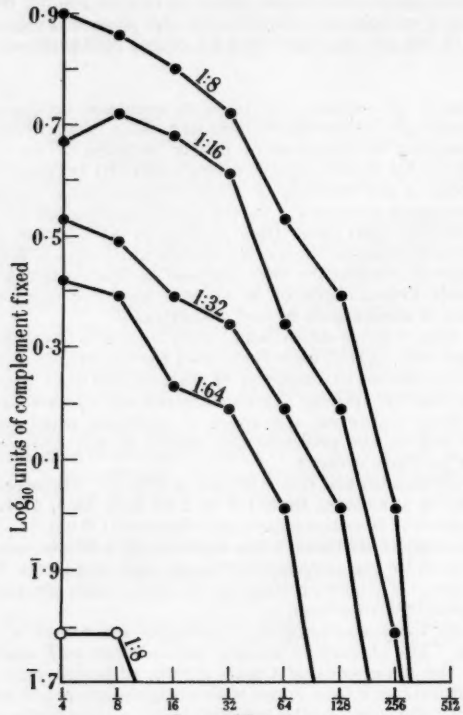

FIG. 2.—Three-dimensional CF test of M antigen and homologous mouse antiserum.

○ = N antigen.
● = M antigen.

Figures on curves refer to antigen concentrations.

Note.—The serum was inactivated at 56° for 30 min.

Recording of results.

Reading.—In each line the square on the perspex plate in which 50 per cent of the cells remain unlysed is taken as the end-point. This point may be estimated to fall half-way between two squares, one of which shows complete lysis and the other no lysis at all. If the proportion of cells remaining unlysed in a particular square is nearer to 25 or to 75 per cent than to all, none or half, the 50 per cent end-point is calculated accordingly. The degree of fixation in individual squares may, therefore, also be expressed by the conventional system of notation, with plus signs, from 0 to 4 (*cf.* Table III).

Calculation.—The assessment of the unit of complement has already been considered. The units fixed are plotted as $\log_{10}$ units along the ordinate on mm.-ruled graph paper,

each centimetre representing 0·1 $\log_{10}$ unit. The serum dilutions are plotted at cm. intervals along the abscissa. The lower limit of the "area of fixation" was taken as the $\bar{1}{\cdot}7$ $\log_{10}$ unit line; in general, irregularities in curves, presumed due to experimental error, were confined below this level. Areas of fixation were estimated by geometrical measurement.

RESULTS.

Fixation of complement by antigen and homologous antiserum.

M antigen was first tested against a mouse anti-Lansing hyperimmune serum with a neutralizing titre against the Armstrong strain of 1/1000. The titre in this instance was defined as the final dilution of serum which neutralized 99 LD_{50} of virus after incubation of equal volumes of serum dilutions and virus suspension at room temperature for 1 hr. and subsequent intracerebral inoculation of mice with 0·03 ml.

A three-dimensional test of the as yet uncharacterized M antigen and a parallel test with N antigen gave results similar to those shown in Fig. 2 for an MEF_1 antiserum. Complement was specifically fixed, the highest serum titre (1/256) at the $\bar{1}{\cdot}7$ $\log_{10}$ unit level of fixation being given by antigen concentrations of 1/8 and 1/16. The curve of fixation with the M antigen was clearly of a different order from that with the N antigen, of which only the 1/8 concentration showed any significant fixation.

Specificity.

The specificity of the reaction is shown in Fig. 3, which records a test with N, M and FA antigens against normal, "control," anti-Lansing and anti-FA sera. Because the FA antigen was being used as a control antigen of infected and inflamed mouseling brain tissue, it was used at the same concentration (1/16) as the M antigen. The sera were from cotton-rats. The two virus antigens fixed complement strongly with their homologous antisera but reacted no more than did the N antigen with the other sera.

Reproducibility.

The variation in amount of complement fixed on any one occasion did not usually exceed 5 per cent on either side of the mean.

Table II shows that tests done at different times gave more variable results than those obtained by Fulton and Dumbell (1949) with the more strongly complement-fixing influenza virus systems with which the variation was not usually greater than 5 per cent on either side of the mean value. In the example given,

TABLE II.—*Reproducibility of the Two-dimensional Test. Comparison of Areas of Fixation with M Antigen and Lansing Cotton-rat Serum Tested on Different Occasions.*

Date.	Area in cm.²	Mean.	Areas referred to mean.
23.i.52	31·6	32·8≡1·00	0·96
12.ii.52	30·2		0·92
21.iii.52	36·6		1·12
1.iv.52	32·8		1·00

The areas are reckoned from the 1 in 16 serum dilution as this was, in one case, the first dilution used. M antigen used at 1 in 16.

the variation was from − 8 to + 12 per cent, but in other tests was greater than this, though seldom more than ± 15 per cent.

Tests of hyperimmune sera.

Hyperimmune sera were produced in mice, cotton-rats and hamsters by the procedure already described. The serum of each species fixed complement

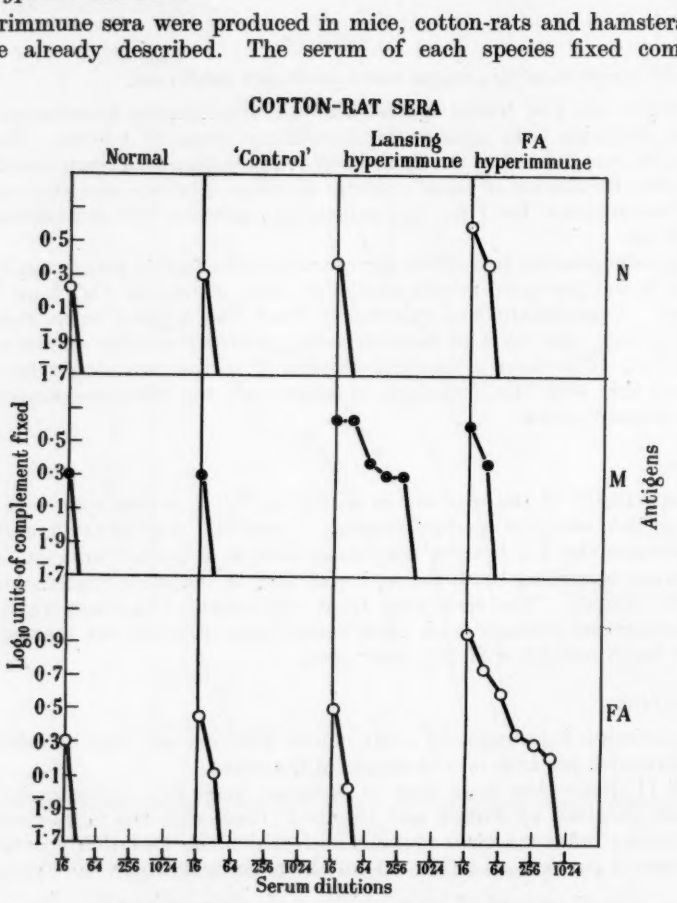

FIG. 3.—Specificity of the CF reaction between M antigen and homologous antiserum.

specifically with the M antigen. The degree of fixation varied with the different sera but was always greater than that obtained with M antigen and normal or " control " serum, or with N antigen in the presence of the hyperimmune serum.

Guinea-pigs were similarly inoculated but their serum did not acquire detectable CF antibodies against type 2 poliomyelitis virus.

Sera were obtained from monkeys inoculated with one or other of the three prototype poliomyelitis viruses mixed with adjuvant. Judging from the results

of a few tests using only the type 2 antigen (Table III), the development of CF antibodies in rhesus monkeys after intramuscular immunization seems to be type-specific; this agrees with the general conclusion of other investigators (Casals, Olitsky and Anslow, 1951*a*; Svedmyr, Enders and Holloway, 1952; Pollard, Hsiang and Sharp, 1952).

With several supposedly normal monkey sera there was a slightly greater fixation with M than with N antigen, amounting to a difference of about 1 +, seldom more. This is presumably of no specific significance. It was mostly seen with sera inactivated at 56° for 30 min., rarely with those heated at 60° for 20 min. At the time of the tests recorded in Table III monkey sera were still being inactivated at the lower temperature but this was later replaced by

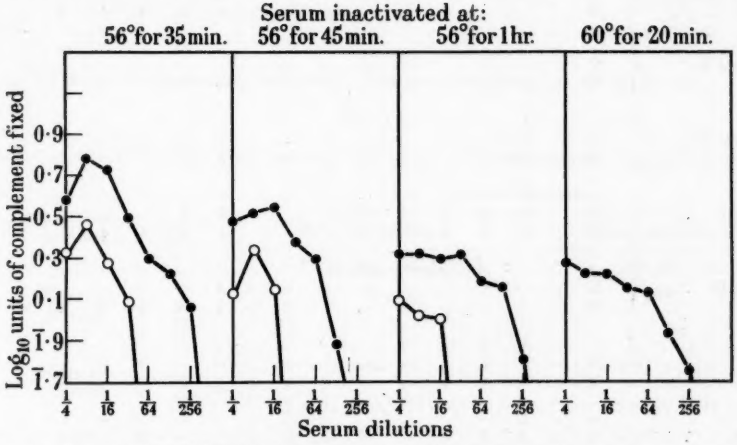

FIG. 4.—Inactivation of Lansing hyperimmune monkey serum under varying conditions.
○ = N antigen.
● = M antigen.

inactivation at 60° for 20 min., as advocated by Casals. The curves of fixation given by a Lansing hyperimmune monkey serum after inactivation in varying conditions of time and temperature are shown in Fig. 4. The higher temperature abolished non-specific fixation. A zone of apparently diminished fixation was seen in the highest serum concentration after heating at 56° for 35 or 45 min. One reason for this was found to be the incomplete inactivation of complement (or of some procomplementary substance) present in the monkey serum.

Tests with convalescent sera from animals.

The pooled serum of hamsters which had survived a paralytic infection with "adapted" MEF_1 virus gave a definite area of specific fixation over and above some non-specific fixation with N antigen. The areas of fixation were: with M antigen, 20·3 cm.^{2}, with N antigen, 3·3 cm.^{2}

The sera of two rhesus monkeys convalescent from intracerebral infection with Lansing virus were examined in line tests (Table IV). Both sera gave some

non-specific fixation, but also greater fixation with M antigen. With serum of monkey I the difference was 2 + which is only suggestive, but with that of monkey II the difference (4 +) was more marked. The serum of the first monkey was taken 19 days, that of the second 5 weeks, after infection.

TABLE III.—*Line CF Tests with Monkey Hyperimmune Sera.*

Serum.	Antigen.	Initial serum dilutions (reciprocals). Pre-immunization. 4.	8.	16.	32.	64.	128.	—	Post-immunization.* 4.	8.	16.	32.	64.	128.	256.	—
MEF_1 hyperimmune mouse serum	—	..							—	—	—	—	—	..	..	..
	N	..							1	—	—	—	—	—	—	—
	M	..							4	4	4	4	3	—	—	—
Brunhilde I	—	1	±	—	—	—	..	..	—	—	—	—	—	..	..	..
	N	1	±	—	—	—	—	—	—	—	—	—	—	—	—	—
	M	2	1	—	—	—	—	—	—	—	—	—	—	—	—	—
Brunhilde II	—	1	—	—	—	—	..	..	—	—	—	—	—	..	..	..
	N	2	±	—	—	—	—	—	4	4	1	—	—	—	—	—
	M	2	1	—	—	—	—	—	4	4	1	—	—	—	—	—
Lansing I	—	4	4	2	—	—	..	..	—	—	—	—	—	..	..	..
	N	4	4	2	—	—	—	—	3	1	—	—	—	—	—	—
	M	4	4	2	—	—	—	—	4	4	4	4	3	1	—	—
Lansing II	—	No longer available							—	—	—	—	—	..	..	..
	N								4	1	—	—	—	—	—	—
	M								4	4	4	4	4	4	1	—
Leon I	—	4	4	—	—	—	..	..	—	—	—	—	—	..	..	..
	N	4	4	±	—	—	—	—	—	—	—	—	—	—	—	—
	M	4	4	1	—	—	—	—	±	—	—	—	—	—	—	—
Leon II	—	2	—	—	—	—	..	..	—	—	—	—	—	..	..	..
	N	4	3	—	—	—	—	—	±	—	—	—	—	—	—	—
	M	4	4	±	—	—	—	—	1	—	—	—	—	—	—	—

* Blood taken 12 weeks after first immunizing injection in case of Brunhilde and Leon monkeys; 10 weeks later in case of Lansing monkeys.
The amount of complement used was 3·0 units.
The monkey sera were inactivated at 56° for 30 min.
The numbers 4, 3, 2 and 1 represent 100, 75, 50 and 25 per cent fixation respectively; ± = a trace of unlysed cells, — = no fixation, .. = not done.

Pre- and post-inoculation sera of monkeys inoculated with different samples of sewage and of one inoculated with the faeces from a case of poliomyelitis were examined (Table V). Two of the sewage samples, in monkeys 154/51 and 155/51, stimulated no detectable CF antibodies. The brains and cords of these monkeys were negative on histological examination. Serum was also obtained from two monkeys inoculated with a single specimen of sewage from a community in which infection was known to exist. The serum of the first (163/51), which was killed when it became paralysed on the 16th day after inoculation, was strongly anti-complementary and no specific fixation was detectable. Serum of the second monkey (164/51), killed on the 24th day after inoculation with the same sewage, exhibited a definite increase in fixation with M antigen. Two days earlier, this monkey had had a temperature of 104° F and tremor. It showed characteristic poliomyelitic lesions of the central nervous system.

TABLE IV.—*Line CF Tests with Monkey Convalescent Sera.*

Serum.	Antigen.	Serum dilutions. Pre-inoculation. 4.	8.	16.	32.	64.	128.	—	Convalescent. 4.	8.	16.	32.	64.	128.	—
Normal rhesus monkey serum (pool)	—	1	—	—	—	—	..	..							
	N	3	±	—	—	—	—	—							
	M	3	1	±	—	—	—	—							
Lansing I	—	4	4	3	—	—	..	..	±	—	—	—	—	..	..
	N	4	4	4	—	—	—	—	4	4	4	—	—	—	—
	M	4	4	4	—	—	—	—	4	4	4	2	—	—	—
Lansing II	—	No longer available							1	—	—	—	—	..	..
	N								4	4	3	—	—	—	—
	M								4	4	4	3	±	—	—

2·6 units of complements were used. Sera were inactivated at 56° for 30 min.

TABLE V.—*Line CF Tests with Sera of Monkeys Inoculated with Faeces or Sewage.*

Serum.	Material injected.	Antigen.	Serum dilutions. Pre-inoculation. 4.	8.	16.	32.	64.	128.	—	Post-inoculation. 4.	8.	16.	32.	64.	128.	—
154/51	Negative sewage	—	4	4	—	—	—	..	..	—	—	—	—	—	..	..
		N	4	4	—	—	—	—	—	—	—	—	—	—	—	—
		M	4	4	—	—	—	—	—	—	—	—	—	—	—	—
155/51	Negative sewage	—	4	4	1	—	—	..	..	—	—	—	—	—	..	..
		N	4	4	±	—	—	—	—	—	—	—	—	—	—	—
		M	4	4	±	—	—	—	—	—	—	—	—	—	—	—
158/51	Stool of case	—	Not available for testing							4	1	—	—	—	..	..
		N								4	1	—	—	—	—	—
		M								4	3	±	—	—	—	—
163/51	Positive sewage	—	Not available for testing							4	4	4	1	—	..	..
		N								4	4	4	±	—	..	..
		M								4	4	4	1	—	—	—
164/51	Positive sewage	—	4	—	—	—	—	..	..	—	—	—	—	—	..	..
		N	4	—	—	—	—	—	—	1	±	—	—	—	—	—
		M	4	1	—	—	—	—	—	4	3	1	±	—	—	—

2·9 units of complement were used. The sera were inactivated at 60° for 20 min.

Monkey 158/51, which had been inoculated with the stool of a patient with poliomyelitis, showed a rise in temperature 10 days later with the onset of tremor on the 18th day and was killed on the 30th day without having developed paralysis. The serum taken at this time showed a slight excess of specific over non-specific fixation. The cord showed lesions typical of poliomyelitis.

Tests of human sera.

Line tests (with 2·5 units of complement).—Four sera from children aged between 1 and 2 years, with no history of poliomyelitis, gave uniformly negative results starting at a dilution of 1 in 4. Similarly, sera from apparently healthy adults

were negative, or almost so, at 1 in 4 ; two of these sera were anticomplementary when tested at 1 in 2. The results obtained with these two and with four other normal sera are shown in Table VI.

TABLE VI.—*Line CF Tests with "Non-poliomyelitic" Human Sera.*

A. SERA FROM HEALTHY ADULTS.

Serum.	Antigen.	Serum dilutions. 2.	4.	8.	16.	32.	64.	—
B 4	—	..	—	—	—	—	—	..
	N	..	—	—	—	—	—	—
	M	..	—	—	—	—	—	—
B 13	—	..	—	—	—	—	—	..
	N	..	—	—	—	—	—	—
	M	..	—	—	—	—	—	—
B 19	—	..	—	—	—	—	—	..
	N	..	—	—	—	—	—	—
	M	..	—	—	—	—	—	—
B 20	—	3	—	—	—	—	—	..
	N	3	—	—	—	—	—	—
	M	2	—	—	—	—	—	—
B 21	—	1	±	—	—	—	—	..
	N	1	—	—	—	—	—	—
	M	1	±	—	—	—	—	—
B 23	—	..	—	—	—	—	—	..
	N	..	—	—	—	—	—	—
	M	..	—	—	—	—	—	—

B. SERA GIVING A POSITIVE WR.

Serum.	Antigen.	Serum dilutions. 2.	4.	8.	16.	32.	64.	—
B 7	—	3	—	—	—	—	—	..
	N	4	4	3	—	—	—	—
	M	4	4	2	—	—	—	—
B 10	—	—	—	—	—	—	—	..
	N	3	1	—	—	—	—	—
	M	2	1	—	—	—	—	—
B 18	—	3	—	—	—	—	—	..
	N	4	4	4	2	—	—	—
	M	4	4	4	2	—	—	—
B 25	—	—	—	—	—	—	—	..
	M	4	1	—	—	—	—	—
	M	4	4	±	—	—	—	—
B 26	—	..	—	—	—	—	—	..
	N	..	—	—	—	—	—	—
	M	..	—	—	—	—	—	—
B 27	—	..	—	—	—	—	—	..
	N	..	±	—	—	—	—	—
	M	..	±	—	—	—	—	—

2·5 units of complement were used. Sera in group A were inactivated at 56° for 30 min., those in group B at 60° for 20 min.

.. = not done.

Various WR-positive sera were also tested with unextracted antigens. The results with six of them are given in Table VI. They showed variable non-specific reactions. Over and above a moderate non-specific reaction, serum B 25 repeatedly gave a clear reaction with M antigen. The question of WR-positive sera is discussed later.

Several paired sera were tested from cases diagnosed clinically as poliomyelitis or in which poliomyelitis was considered in the differential diagnosis (Table VII).

TABLE VII.—*Line CF Tests with Sera from Cases of Poliomyelitis or ? Poliomyelitis.*

Serum.	Diagnosis.	Age of patient (years).	Antigen.	Serum dilutions. First specimen. 4.	8.	16.	32.	64.	128.	—	Second specimen. 4.	8.	16.	32.	64.	—
MEF_1 hyper-immune mouse serum			—	—	—	—	—	..	..							
			N	3	—	—	—	—	—	—						
			M	4	4	4	4	4	±	—						
4084	? Non-paralytic poliomyelitis	5	—	—	—	—	..	..	..	..	—	—	—	..	..	..
			N	—	—	—	—	—	..	—	—	—	—	—	—	—
			M	—	—	—	—	—	..	—	±	—	—	—	—	—
4085	Poliomyelitis	13	—	±	—	—	..	..	..	..	—	—	—	..	..	..
			N	±	—	—	—	—	..	—	—	—	—	—	—	—
			M	1	±	—	—	—	..	—	—	—	—	—	—	—
4090	Poliomyelitis	20	—	—	—	—	..	..	..	..	—	—	—	..	..	..
			N	—	—	—	—	—	..	—	—	—	—	—	—	—
			M	3	±	—	—	—	..	—	±	—	—	—	—	—
4143	Poliomyelitis	3½	—	—	—	—	..	..	..	..	3	—	—	..	..	..
			N	—	—	—	—	—	..	—	3	—	—	—	—	—
			M	±	—	—	—	—	..	—	4	3	1	—	—	—
4144	Poliomyelitis	5½	—	—	—	—	..	..	..	..	—	—	—	..	..	..
			N	—	—	—	—	—	..	—	—	—	—	—	—	—
			M	1	—	—	—	—	..	—	±	—	—	—	—	—
4145	Abortive poliomyelitis	11	—	—	—	—	..	..	..	..	—	—	—	..	..	..
			N	—	—	—	—	—	..	—	—	—	—	—	—	—
			M	—	—	—	—	—	..	—	—	—	—	—	—	—
A 1	? Non-paralytic poliomyelitis	31	—	3	—	—	..	..	..	..	—	—	—	..	..	..
			N	2	—	—	—	—	..	—	—	—	—	—	—	—
			M	4	2	—	—	—	..	—	—	—	—	—	—	—
A 4	Poliomyelitis	7	—								—	—	—	..	..	..
			N	Not suitable for use							—	—	—	—	—	—
			M								4	1	—	—	—	—

2·9 units of complement were used. Human sera inactivated at 60° for 20 min. With the exception of sera A 1 and A 4, the first specimen was collected during the first week of illness, the second between 3 and 6 weeks later.

Of the first six pairs of sera the first specimen of 4090 and the second of 4143 gave definite specific fixation; two other first specimens gave a doubtful positive result. An explanation of these few results is discussed below. Serum 4090a was also tested at a dilution of 1 in 2 and did not fix complement by itself or in the presence of N antigen, but gave 4 + fixation with M antigen.* Sera which

* Since this paper was completed, a strain of poliomyelitis virus from the stool of patient 4090 has been isolated and typed in tissue culture by Dr. A. P. Goffe and Miss J. J. Beveridge. It was found to be of type 3.

can be tested at 1 in 2 will clearly yield better information than those which can not.

Serum from a traveller recently returned from the Middle East, which had been stored at − 20°, showed a moderate excess of specific over non-specific fixation, although there was no history of poliomyelitis; whereas a specimen taken over a year later, during an illness diagnosed clinically as non-paralytic poliomyelitis, showed no reaction at all (see A1 in Table VII).

A single specimen of serum (A4 in Table VII) was obtained during the 3rd month of convalescence from a child of 7 from whose stool virus had been isolated.* The serum had an appreciable content of type 2 CF antibody. (The serum findings of monkey 158/51, inoculated with this child's stool, have been mentioned above, *cf.* Table VI, as have those of the monkeys inoculated with sewage from the community in which the child lived.)

Restricted two-dimensional tests.—Some of the sera referred to above were further examined in the limited form of two-dimensional test described on p. [303]. The differential fixation with M antigen demonstrable at the single complement level corresponded fairly well with the area of specific fixation given by the same serum in the two-dimensional test (*cf.* Table VII and Fig. 5). The advantages of this form of test are discussed later.

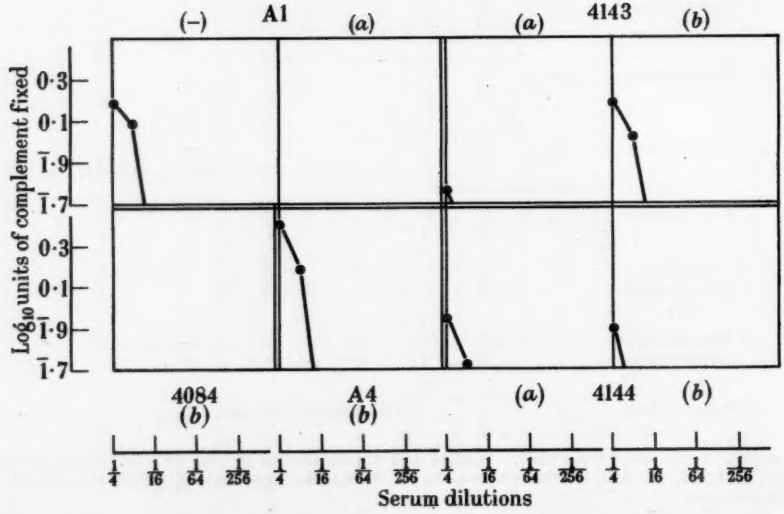

FIG. 5.—Two-dimensional CF tests of human sera. The sera were inactivated at 60° for 20 min.

(*a*) = first specimen.
(*b*) = second specimen.
(−) = specimen taken before onset of illness.

(*cf.* Table VII.)

(Results are those obtained with M antigen: with N there was no significant fixation.)

* Virus isolated from this patient's stool has since been found to be of type 2.

Inactivation experiments.

Methods of rendering the unextracted type 2 antigen non-infective were investigated. The agents used were heat and ultra-violet light (UVL). In each case N antigen was subjected to the same treatment as the M antigen. The results of these experiments are depicted in Fig. 6.

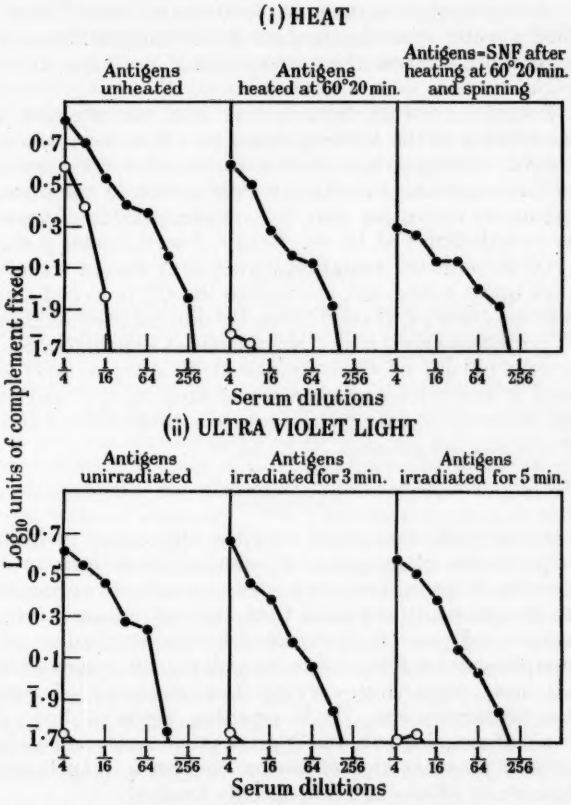

FIG. 6.—Inactivation of M antigen by heat and ultra-violet light.

○ = N antigen.
● = M antigen.

Note.—The same hamster MEF_1 hyperimmune serum was used in both experiments, but in the heating experiments it had been inactivated at 56° for 30 min., whereas in the UVL experiments it was inactivated at 60° for 20 min.

Heat.—Antigen in a concentration of 1/8 or less was placed in a test-tube and heated in a water-bath at either 56° for 30 min. or 60° for 20 min. This made it milky, but the antigen proved nevertheless to be still quite effective. The reading of end-points, though not so immediately clear as with the ordinary antigen, turned out to be fairly straightforward when aided by a little judicious

tapping of the plates. The coagulated material usually collected as a thin veil in the centre of the drop with the button of red cells lying immediately beneath it on the surface of the perspex.

Heating to 56° or 60° caused a moderate reduction in the efficacy of the M antigen, as tested with type 2 hyperimmune antiserum, and a similar or greater decrease in the amount of fixation given by N antigen with the same serum. Centrifugation of the heated antigen at 4000 r.p.m. for 15 min. in an angle centrifuge yielded a water-clear supernatant fluid which still gave a considerable area of specific fixation, whereas the corresponding N antigen failed to fix complement altogether.

M antigen heated at either temperature was non-infective, as judged by intracerebral inoculation of the 1/8 suspension into 3- to 4-week-old mice.

Ultra-violet light.—Antigen in a concentration of 1/8 in veronal-saline was placed in 0·5 to 1 ml. amounts in ordinary Petri dishes (9 cm. diam.) and rocked at the rate of about 30 cycles per min. for varying periods of time. The centre of the dish was at a distance of 10 cm. from a 7-watt, water-jacketed mercury-vapour lamp. Of the emitted radiation 95 per cent was of wavelength 2537 A.

Irradiation for up to 5 min. did not reduce the CF power of the antigen. In Fig. 6 are shown the areas of fixation given by unirradiated antigen and antigen treated with UVL for 3 and for 5 min. No obvious change in fixation is detectable.

Inoculation of 0·03 ml. of UVL-irradiated M antigen (1/8) intracerebrally into mice showed it to be incompletely inactivated at 1, 2 and even 3 min. in these conditions, whereas no infectivity was detectable after 5 min. The usual LD_{50} titre of M antigen lies between 10^{-5} and 10^{-6}.

DISCUSSION.

This paper is principally concerned with the application of Fulton and Dumbell's CF technique to the examination of poliomyelitis antigen-antibody systems. The method of using drops on perspex plates not only saves reagents but makes feasible tests more spaciously designed than the conventional form of tube test in which the serum end-point is determined with a single dose of complement. Where little complement is fixed, as with poliomyelitis convalescent sera, the two-dimensional test, employing varying concentrations of both serum and complement, has certain merits. It is sensitive, since it shows fixation of as little as half a unit of complement, and it gives greater assurance of the specificity of the result since it provides at each serum dilution a quantitative estimate of any anticomplementary effects and non-specific fixation.

The two-dimensional test may help in studying changes in the specificity of poliomyelitis antigens. Casals, Olitsky and Sabin (1952) have shown that in human beings the CF antibody response to poliomyelitis virus, as tested with the type 2 mouseling brain antigen, is less type-specific than the neutralizing antibody response.* However, even in complement-fixation tests some human poliomyelitis sera fail to react with type 2 antigen, either because the patient does not produce sufficient antibody for long enough or because the antibody produced is unusually type-specific (*cf.* Table VII). It will be worth while seeking a

* Since this paper was accepted for publication Svedmyr, Enders and Holloway have published a fuller account of their experiments with poliomyelitis virus antigens of types 1, 2 and 3 prepared from tissue cultures (1953: *Amer. J. Hyg.*, **57**, 60). Their results provide further evidence of the broad character of the poliomyelitis CF antibody response in human beings.

modified type 2 CF antigen, still derived from mouseling brains, but with its specificity altered by chemical, physical or perhaps biological manipulation in such a way as to make it react regularly with antibodies against all three types of poliomyelitis virus.

It seems justifiable, for the present, to continue using the unextracted M antigen in tests of human sera in view of its easier preparation. Serum B25 in Table VI, a WR-positive serum, fixed more complement with M antigen than with N antigen. Although this serum came from a patient with no history of poliomyelitis, it is probable that the greater reaction with M antigen was due to poliomyelitis antibodies. It seems therefore that even WR-positive sera and others giving non-specific fixation can sometimes be tested with unextracted antigens. In any case such sera are unlikely to be common in this country among those sent for testing. This matter requires further study, but when unextracted antigens give an equivocal answer it is still possible to re-test with acetone-ether extracted antigens.

SUMMARY.

The CF technique of Fulton and Dumbell, using drops of reagents on perspex sheets, has been successfully applied to poliomyelitis antigen-antibody systems. The different ways of performing the test are described in detail.

The antigen was an aqueous suspension of mouseling brains infected with the " adapted " MEF_1 strain of type 2 poliomyelitis virus; unlike that of Casals and Olitsky, it was not acetone-ether extracted.

In two-dimensional CF tests, with varying concentrations of complement and serum, as in conventional one-dimensional tests with a single complement dose, specific fixation was obtained with the unextracted antigen and hyperimmune Lansing and MEF_1 (type 2) antisera. No specific fixation was detected with hyperimmune monkey sera against type 1 and type 3 poliomyelitis viruses.

A few human sera were examined. In general, those from children and adults with no history of recent poliomyelitis showed no fixation with the unextracted poliomyelitis antigen although some WR-positive sera gave variable non-specific reactions. Paired sera from patients mostly showed slight, but in some cases definite, fixation with the type 2 antigen in one or other specimen.

Heating at 56° for 30 minutes, or at 60° for 20 minutes, destroyed the infectivity of the antigen for mice, as did ultra-violet irradiation for 5 minutes. Heating reduced the titre slightly, whereas this amount of irradiation did not.

The MEF_1 " adapted " strain was kindly supplied by Dr. Jordi Casals, the Lansing strain by Dr. Joseph L. Melnick and the FA strain by Dr. Max Theiler.

I am indebted to Dr. F. O. MacCallum and to Dr. Alan Goffe for details of the monkey experiments mentioned in this paper.

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GENETIC INTERACTION AND INTERFERENCE BETWEEN THE MEL AND NWS STRAINS OF INFLUENZA A VIRUS.

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RECENT work by Fraser and Burnet (1952) and Burnet and Lind (1952) has substantiated Burnet's hypothesis that neurotropic forms of the MEL strain of Influenza A, recovered from mixed infections with non-neurotropic MEL and the neurotropic strain NWS, originate by some form of genetic interaction akin to recombination (Burnet and Lind, 1951). By simultaneous inoculation of both virus strains into the allantoic cavity, a fluid was produced containing the product of only one reproductive cycle of mixed infection from which were recovered the two parent strains and the well-characterised N-M virus, the neurotropic derivative of MEL (Burnet and Lind, 1951). As the work first mentioned depends on the validity of certain experimental methods which are involved in the observations reported here, a brief recapitulation of these is necessary. The first was to adapt the technique of Cairns (1952) which, by a method of emptying and washing the allantoic cavity, made available fluids which contained, for the most part, only the product of one reproductive cycle following double infection of the allantois with the Influenza A strains NWS and MEL. These are referred to as one-cycle fluids. The second was to isolate the strains present in such fluids by suppressing with specific antiserum any further cycles of NWS and inoculating the neutralised fluid on the chorio-allantoic membrane of the chick-embryo. The third was to identify, by the method of limit dilution, the causative organism in the brain of chick-embryos showing typical haemorrhagic lesions after such inoculation. This was invariably found to be a strain of the N-M type. The fourth was to establish the fact that such strains did not appear using the same technique and materials, when the starting fluid was a simple mixture of MEL and NWS as opposed to a one-cycle fluid from mixed infection. In such circumstances N-M appeared only when NWS was present in a quantity great enough to escape the neutralising action of the specific antiserum. (The recovery of mouse-pathogenic N-M strains after chorio-allantoic inoculation of NWS and MEL had already been reported by Burnet and Fraser (1952).) It was therefore concluded that N-M originated during the first cycle of double infection in the allantoic cavity and not at a later stage in the course of isolation. This conclusion was rapidly confirmed by Burnet and Lind (1952). When these authors used WSE and MEL strains as parent viruses in the same way two new strains of virus were isolated from the one-cycle fluids, each related serologically to one of the parent strains and illustrating a reciprocal transfer of properties between WSE and MEL. In this instance the yield of recombinant strains was so great that

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the method of limit dilution was the only step necessary to their isolation from one-cycle fluids. Antiserum was not required.

Experiments were next undertaken to examine what degree of coincidence of the reproductive cycles of the parent strains was essential to the recovery of N-M virus. As N-M almost certainly originates by double infection of the same cell, its appearance ought to bear some relation to variations in the time interval between the inoculation of NWS and of MEL into the allantoic sac. The NWS-MEL system has the disadvantage of requiring two successive titrations to accomplish the isolation and the identification of N-M and might well have been superseded, for the purposes of this investigation, by the more readily handled WSE-MEL combination of Burnet and Lind (1952), but the former was chosen because of two observed differences from the results obtained with WSE and MEL. These were the failure to isolate a non-neurotropic form of NWS and the fact that N-M frequently failed to appear after the inoculation of large doses of NWS into the allantoic cavity. Lest this should be a reflection of some fundamental difference in the interaction of the two pairs of parent strains, the NWS-MEL system was investigated first.

MATERIALS AND METHODS.

Virus.—The strains used in all the experiments were (1) the standard Influenza A strain, MEL (Burnet, 1935), not normally pathogenic for the chick embryo and (2) the strain NWS (Stuart Harris, 1939), a neurotropic variant of the WS strain of Influenza A, and pathogenic for the chick embryo. A stock of each strain was prepared by allantoic inoculation of a number of eggs with 10 ID_{50} of the strain, and pooled positive fluids from the respective strains were stored in ampoules in dry ice. The content of each pool was, MEL 400 AD $10^{8\cdot5}$ ID_{50} per 0·05 ml. and NWS 140 AD, $10^{6\cdot8}$ ID_{50} per 0·05 ml. Fresh ampoules of the stocks were thawed for each experiment.

RDE.—A semi-purified preparation of this enzyme of *V. cholerae* was used.

Haemagglutination.—This refers to the agglutination of 1 per cent fowl cells in saline, in volumes of 0·25 ml. of each reagent, read to a " one plus " end-point after 40 min. at room temperature.

Antisera.—These were immune ferret sera prepared against the actual strains used in the experiments. They were used for neutralisation of infectivity and for anti-haemagglutinin titrations in dilutions which had been shown not to cross-react with the heterologous strain of the two in use.

Production of one-cycle fluids.—The method of Cairns (1952) was used with the differences described by Fraser and Burnet (1952) and in this text.

Estimation of N-M content.—The titre was estimated from a count of the number of embryos showing specific lesions four days after the chorio-allantoic inoculation of dilutions of the one-cycle fluid in a 1/25 dilution of anti-NWS serum. Groups of four or groups of six eggs were used per dilution. Incubation was at 35·5°. The identity of the lesion-producing virus was always checked by sub-allantoic propagation to limit dilution of the emulsions of two embryo brains taken from the upper limit of the chorio-allantoic titration. Allantoic fluids from these limit dilutions were identified by the resistance of their haemagglutinin to heat, by their serological reaction and by their convertibility to the indicator state on simple heating at 56°. In a series of eleven experiments of this type, N-M was the only virus recovered from embryos showing specific lesions. NWS did not break through the protective antiserum of the chorio-allantoic titration.

RESULTS.

Preliminary experiments.

A series of preliminary experiments in embryos from which the allantoic fluid had not been removed, produced irregular results which showed, by com-

parison with all those done later after removal of the allantoic fluid, that this fluid, perhaps by temporary deviation of a portion of the inoculum, could produce two different effects. With small inocula of NWS—up to 0·5 ml. of undiluted seed virus—the interval between the two inocula, NWS and MEL, could be prolonged to four hours and N-M still appear in the one-cycle fluid. Also, in the presence of allantoic fluid, the recovery of N-M was not correlated with the dosage of NWS.

When the technique of preliminary removal of allantoic fluid had been adopted, a series of experiments in which the two viruses were introduced simultaneously was undertaken in order to determine the relative dosage which gave the greatest yield of N-M. A clear-cut answer was obtained. With the two particular seed viruses in use and a constant inoculum of MEL of 0·05 ml. of a 1/10 dilution of stock virus, the optimum dose of NWS was 0·05 ml. of undiluted stock virus. This gave a maximum titre of N-M of $10^{2.5}$ ID_{50} per 0·05 ml. of one-cycle fluid. Occasionally N-M was recovered following the inoculation of 0·5 ml. of undiluted seed, none after 1·0 ml., 2·0 ml. or 5·0 ml. The time-interval experiments were therefore standardised with the optimum dosage of NWS.

The standard experiments.

For reasons which appear in the text, most attention was paid to the situation where NWS is inoculated before MEL but in all experiments some infections were included in which MEL preceded NWS. Four experiments of the same type were done. Groups of four eggs in the third experiment and of three eggs in the other three experiments were used for each time interval and the term " one-cycle fluid " for any time-interval refers to a pool of equal volumes of allantoic fluid from each egg in the group.

After allantoic fluid had been removed and the cavities flushed with two changes of saline at 37°, inocula were made in the following way : NWS 0·05 ml. in 5·0 ml. of saline at 37°, followed at the chosen times by MEL 0·05 ml. in 1·0 ml. saline at 37°. Two hr. after the second inoculum 1·0 ml. or 1·5 ml. of RDE, about 2000 units, warmed to 37°, was inserted and 1 hr. later the cavities were emptied, washed 4 times in not less than 8·0 ml. of warm saline and the contents replaced by 5·0 ml. of saline at 37°. To equate volumes, the simultaneous dose of NWS and MEL was given in 6·0 ml. of warm saline. When MEL preceded NWS, rapid distribution of the small inoculum was ensured by rotating the egg several times. An incubation period of 8 hr. after the second inoculum was chosen in each experiment, a period long enough to permit the appearance of the major part of the product of the reproductive cycle of the second inoculum, but not so long as to allow the completion of any second cycle by the first inoculum. A consequence of the method is that the time available for the absorption of the strain first inoculated is necessarily longer than that available to the second. Also, it was not feasible to extend the time between the two inocula beyond four hr., as that time plus the three hr. necessary for the absorption of the second virus and for the action of RDE, would exceed the period when new virus from the primary inoculum begins to appear in the allantoic fluid.

At the end of 8 hr. incubation at 37° fluids were harvested, pooled and stored in dry ice for subsequent examination. Alternatively, the groups of eggs were chilled rapidly at −15° for 30 min. then stored overnight at 4°, to be harvested and pooled on the following morning.

In tabulating results the moment of inoculation of MEL in each group is 0·0 hr. The time of inoculation of NWS is given as minus or plus x hr. with reference to 0·0 hr.

Serological analysis of one-cycle fluids.

The inocula used in these experiments were large enough to produce measurable amounts of haemagglutinin in one reproductive cycle. Serological examination of the fluids was carried out in all four experiments—for each separate egg in experiments 1, 2 and 4—by titration of the fluids in saline and in a high dilution of each antiserum. The results were not only in agreement with the infectivity titrations of experiments 3 and 4, but also provided information about the character of certain one-cycle fluids which could not be derived from infectivity titrations. This method of titration allows analysis of the content of one-cycle fluids in terms of their serological components. When these components have either one character (NWS) or the other (MEL), as in simple artificial mixtures, the behaviour of the fluids is absolutely predictable and the amounts of haemagglutinin of either sort can be estimated with reasonable accuracy, as for example in Table I. This may be compared with the behaviour of one-cycle fluids in

TABLE I.—*The Titration of Artificial Mixtures of NWS and MEL in Saline and in Dilute *Antisera to NWS and MEL.*

Fluid.	Composition of Mixture, AD/0·05 ml.		Haemagglutinin titre in—		
	MEL.	NWS.	Saline.	Anti-MEL.	Anti-NWS.
1	24	20	28	8	12
2	24	10	28	6	12
3	24	5	20	3	12
4	24	2½	20	1½	12
5	24	0	20	0	12
6	24	20	32	10	14
7	12	20	28	12	7
8	6	20	20	12	1½
9	3	20	20	12	½
10	0	20	20	10	0

The sum of the titres in the two antisera is one-half the sum of the two components. Throughout, the titres of the eight mixtures in saline are less than the sum of the components.

* Dilutions of anti-MEL 1/500 and anti-NWS 1/300 were used for all these and subsequent tests.

Table II which summarises an experiment carried out in the manner of a standard time-interval experiment, but using large doses of NWS and of MEL (2·0 ml. and 0·1 ml. of undiluted stock virus respectively) in order to obtain large amounts of haemagglutinin. The titres given are the mean titres per group of three eggs. The characteristics of these fluids are precisely those of the small quantities produced in the standard experiments (Tables III and IV) and the change in the production from one type of haemagglutinin to the other is as sharp and occurs at about the same time interval. The significant features of Tables I and II are:

(1) The titre of an unmixed fluid in heterologous antiserum is one tube less than in saline.

TABLE II.—*The Production of Anomalous Haemagglutinin with Large Inocula of NWS and MEL.*

Egg Nos.	Time of NWS inoculum.	Haemagglutinin titre in—			Type of haemagglutinin.	N–M titre.
		Saline.	Anti-MEL.	Anti-NWS.		
1–3	−2 hr.	130	66	<10	NWS	0
4–6	−1 hr.	130	14	13	anomalous	0
7–9	−½ hr.	300	9	30	,,	0
10–12	0 hr.	300	<10	35	,,	0
MEL	..	240	<10	120	MEL	..
NWS	..	160	80	15	NWS	..

Titres are the mean of three fluids per group. The time of inoculation of MEL in all groups is 0 hr. Dosage: NWS 2·0 ml., MEL 0·1 ml., both undiluted stock fluid.
(The use of large inocula accounts for the non-recovery of N–M).

(2) The sum of the titres of an artificial mixture of the two haemagglutinins is one-half the sum of the titres of its two components. It is thus never less than half the titre of the mixture in saline. This follows from point (1) and is borne out by the results for mixtures in all the proportions tested.

(3) The sum of the titres of one-cycle fluids diluted in antiserum is, in all but the longest time interval of Table II, very much less than half the titre of the fluid in saline. This type of haemagglutinin has been designated anomalous haemagglutinin. Its significance will become apparent in the next sections.

Analysis of one-cycle fluids by infectivity titration.

Accepting the limitations on accuracy imposed by the titration of a pooled fluid as compared with estimating the mean titres for each group, three measurements were made on each one-cycle fluid. The content of MEL was estimated by sub-allantoic titration in antiserum to NWS; the content of NWS by chorio-allantoic titration in antiserum to MEL and the N-M content by chorio-allantoic titration in antiserum to NWS. Groups of four eggs were used per dilution, with the occasional use of groups of six eggs for N-M titrations, and end-points calculated according to the Reed-Muench method. End-points for MEL infectivity were always sharply defined; those for N-M were somewhat irregular, but reproducible; those for NWS were very irregular and widely spread. This irregularity in the titration of NWS and N-M may be related to the serological character of the one-cycle fluids (see above), for it was certainly not so apparent in former work (Fraser and Burnet, 1952), when titrating simple mixtures of NWS and MEL by the same procedure. As recorded in Table IV samples of pooled fluids from the fourth washing of each group of eggs were also titrated in order to show that at all time intervals multiplication had occurred. The range of titres of the wash-fluids which were taken well before incubation was completed, is from $10^{2\cdot0}$ ID_{50} to $10^{4\cdot0}$ ID_{50}, that of the fluids after incubation $10^{5\cdot5}$ ID_{50} to $10^{7\cdot8}$ ID_{50}, clear evidence that the lowest amounts of infective virus contained in fluids 91080 ($=10^{5\cdot5}$ ID_{50}) and 91084 ($=10^{6\cdot0}$ ID_{50}) represent virus newly produced in the allantoic membrane and not residual virus from the starting inoculum. The NWS content of the wash fluids was measured in only two samples but may be taken as considerably less than the corresponding titre of MEL.

The second two standard experiments were analysed in this way.

The Relationship between Interference, Serological Character and the Production of N-M.

If reference is made to Tables III and IV two correlations become apparent.

Table III provides an analysis of experiments 1 and 2 by the serological method. In the Table haemagglutinin is designated NWS when the titre of the fluid in antiserum to NWS had been reduced to zero and the titre in antiserum

TABLE III.—*The Relationship between the Yield of N-M and the Serological Type of Haemagglutinin in Experiments 1 and 2.*

Time.	Experiment 1.			Experiment 2.		
	Fluid.	N-M titre.	Serology.	Fluid.	N-M titre.	Serology.
−4 hr.	92049	0	NWS	..	..	..
−2 hr.	92058	$10^{1.0}$	anomalous	92160	0	NWS
−1 hr.	92055	$10^{1.8}$	,,	92163	$10^{0.8}$	anomalous
0 hr.	92061	$10^{2.0}$	,,	92166	$10^{0.5}$	,,
+1 hr.	..	..	..	92169	$10^{0.0}$	MEL
+2 hr.	92052	$10^{2.0}$	MEL	*	0	MEL

* Fluid from one egg only.
$10^{1.0}$ etc. = ID_{50} per 0·05 ml. 0 = No N-M recovered. .. = Not tested.

to MEL had been greater than half the corresponding titre in saline. It shows that, when N-M has been recovered, the haemagglutinin has been of the anomalous type. This refers only to the situation in which the inoculation of NWS has preceded that of MEL. In experiment 2 the dosage of NWS was increased to 0·25 ml. of undiluted seed and of MEL to 0·05 ml. of undiluted seed virus. The increase of dosage explains the low yield of N-M.

In Table IV is presented a detailed analysis, by serological characterisation and by infectivity titrations, of experiments 3 and 4 in both of which the optimum

TABLE IV.—*An Analysis of the Virus Content of One-cycle Fluids, Experiments 3 and 4.*

Fluid.	Time.	Virus content of 4th wash-fluid.		Virus content of one-cycle fluids.			Haemagglutinin titre of one-cycle fluid in—			Serological type.
		NWS.	MEL.	NWS.	MEL.	N-M.	Saline.	Anti-MEL.	Anti-NWS.	
Experiment 3.										
91080	−4 hr.	..	$10^{3.5}$	$10^{3.8}$	$10^{5.5}$	0	16	12	0	NWS.
91084	−2 hr.	$<10^{1.0}$	$10^{4.0}$	$10^{3.8}$	$10^{6.0}$	0	16	8	0	,,
91088	−1 hr.	..	$10^{3.0}$	$10^{4.0}$	$10^{7.5}$	$10^{1.0}$	8	0	0	Anomalous
91092	−½ hr.	..	$10^{3.0}$	$10^{3.0}$	$10^{7.5}$	$10^{1.5}$	16	1½	4	,,
91096	0 hr.	..	$10^{3.0}$	$10^{3.0}$	$10^{7.0}$	$10^{2.0}$	24	1	4	,,
91100	+½ hr.	$<10^{1.0}$	$10^{2.0}$	$10^{3.5}$	$10^{7.0}$	$10^{1.0}$	48	1	32	MEL
Experiment 4.										
91368	−2 hr.	..	..	$<10^{4.0}$	$10^{6.0}$	$10^{1.5}$	16	0	tr.	Anomalous
91371	−1 hr.	..	..	..	..	$10^{1.2}$	20	tr.	3	,,
91374	−½ hr.	..	..	$10^{3.0}$	$10^{7.0}$	$10^{1.5}$	20	0	3	,,
91377	−¼ hr.	..	..	..	..	$10^{1.5}$	20	tr.	3	,,
91380	0 hr.	..	..	$10^{3.5}$	$10^{7.5}$	$10^{1.5}$	16	,,	0	,,
91383	+½ hr.	..	..	$10^{3.0}$	$10^{7.5}$	$10^{1.5}$	8	0	0	,,
NWS	..	..	..	..	..	..	20	10	0	NWS
MEL	..	..	..	..	..	..	24	0	14	MEL

dosage of NWS and of MEL was used. The same relationship between the yield of N-M and the anomalous nature of the haemagglutinin will be seen. Also, in two fluids from which this type of haemagglutinin and N-M were absent, there was a sharp drop in the yield of MEL. By infectivity titration the degree of interference is considered significant if the titre of MEL has dropped by more

than one dilution from the mean of the titres at zero and later times, provided that this is in accord with the serological data. This precaution is included to compensate for any inaccuracies inherent in restricting the infectivity titrations to pooled fluids. It will be observed that in experiment 3 interference has occurred in fluids 91080 and 91084 in which cases the fluid consists of NWS haemagglutinin. Other fluids, up to zero time, were of anomalous serological character. In experiment 4 no demonstrable interference has occurred and all fluids at relevant times contain anomalous haemagglutinin.

Anomalous haemagglutinin is neutralised by antisera specific for the respective parent strains. Whatever the cause of this may be, it is a distinguishing characteristic of the product of mixed infection within certain time limits of inoculation of the separate parent strains. This applies whether both serological components are detectable or not. That is, if the content of one component is below the threshold of agglutination for fowl erythrocytes, the fact that the haemagglutinin present is reduced by heterologous antiserum to well below half its titre in normal saline would seem to indicate anomalous haemagglutinin. Fluid 91092 may be taken as an example in which both types of haemagglutinin are apparent; fluid 91374, an example in which the NWS component does not appear, when titrated in antiserum to MEL, but the agglutinin present is not " pure " MEL virus for its titre is greatly reduced by antiserum to NWS. This serological character was, therefore, taken as evidence of haemagglutinin produced by mixed intracellular infection. Its absence when the intervals between the inoculations of the parent strains were long, which coincides with the non-appearance of N-M, was taken to be an indication of the presence of an overwhelming amount of one type of agglutinin, that is, an indication of interference with the growth of MEL by NWS.

In all titrations in experiments 1, 2 and 4, no single egg produced a fluid which differed in serological character from the other eggs of its group.

Table V is a summary of the four standard experiments. It shows the relationship between the titres of N-M recovered in each experiment and the presence or absence of interference by NWS with the growth of MEL.

TABLE V.—*A Summary of the Data of Four Time Experiments showing the Relationship between the Recovery of N-M and the Onset of Interference with the Growth of MEL.*

Expt. No.		Time of inoculation of NWS in hours. MEL inoculated at 0·0 hr.									
		−4	−2	−1	−½	−¼	0	+¼	+½	+1	+2
1	N-M titre	0	$10^{1\cdot0}$	$10^{1\cdot8}$	..	..	$10^{2\cdot0}$	..	..	..	$10^{2\cdot0}$
	Interference	+	−	−	..	..	−	..	..	..	−
2	N-M titre	..	0	$10^{0\cdot8}$	..	..	$10^{0\cdot5}$	..	..	10^{0}	0*
	Interference	..	+	−	..	..	−	..	..	−	?*
3	N-M titre	0	0	$10^{1\cdot0}$	$10^{1\cdot5}$	..	$10^{2\cdot0}$	..	$10^{1\cdot8}$	..	..
	Interference	+	+	−	−	..	−	..	−	..	..
4	N-M titre	..	$10^{1\cdot5}$	$10^{1\cdot2}$	$10^{1\cdot5}$	$10^{1\cdot5}$	$10^{1\cdot5}$	$10^{1\cdot8}$	..	..	..
	Interference	..	−	−	−	−	−	−	..	..	..

$10^{1\cdot0}$ etc. = ID_{50} per 0·05 ml. 0 = No N-M recovered. + = Interference established. − = No interference detected.

* Only one egg of the group available.

From this table, constructed partly by accepting the serological evidence as indicating the presence or absence of interference, we may deduce that the non-

recovery of N-M is associated with a measurable degree of interference with MEL by NWS. For this to occur the critical interval between the inocula seems to be about two hr., in that recombinants may or may not be recovered at that time-interval, whereas when the interval is one hour or less, N-M is invariably recovered.

DISCUSSION.

The low yield of N-M in this system is a remarkable contrast to the high yields of recombinants in the WSE-MEL experiments of Burnet and Lind (1952). The optimum amounts of infective virus inoculated in the present experiments were NWS $10^{6\cdot8}$ ID_{50} and MEL $10^{7\cdot5}$ ID_{50}, quantities which, according to the very thorough computations of Fazekas and Cairns (1952), are insufficient to cause double infection of all available allantoic cells. This alone might account for the small recovery of N-M.

Another factor must be taken into account. Any increase in dosage is accompanied by a fall in the recovery rate of N-M in spite of an increasing yield of anomalous or of normal haemagglutinin (Table II). It is likely that, in the particular conditions of isolation, the viability of the N-M has diminished. There are three ways in which this may occur. It is possible that nothing resembling the N-M form is produced, a circumstance which the serological data make unlikely. It is possible also that high dosage, leading to multiple infection of single cells, causes the production of so much anomalous haemagglutinin that N-M is produced only in a form which is susceptible to the neutralising action of both antisera. That is, the method of isolation is invalid. We cannot decide against this second possibility except in the unlikely event of finding a fluid containing more N-M than MEL strains thus allowing direct isolation at limit dilution. This has not been found in any fluid tested even after massive dosage of both parent strains. The third and most likely possibility is that incomplete non-infective N-M is being produced instead of infective virus, because of the excessive demands of a large inoculum of NWS on an unidentified host factor (Cairns and Edney, 1952). The readiness with which the growth of MEL at one site in the embryo may prevent lesion-production by NWS inoculated intravenously (Burnet and Fraser, 1952), suggests that NWS is somewhat susceptible to an attack on its nutritional requirements and it is reasonable to suppose that NWS particles competing intracellularly with MEL would fail to complete the full cycle of maturation, when NWS particles growing in cells otherwise unoccupied would succeed.

Be this as it may, the result of these restrictions on dosage is that a considerable number of allantoic cells will, on mathematical grounds, be infected either by NWS alone or by MEL alone. Theoretically the remainder will be infected by both viruses. Thus at all time intervals there is, so to speak, an internal control consisting of the production of NWS and MEL from singly infected cells in the same membrane in the course of the same period of incubation. The onset of interference is therefore the only important development which differentiates fluids which do not contain N-M from those which do. The possibility of N-M arising by some extracellular process during extrusion of the newly produced virus is thus ruled out.

A consequence of the low recovery rate of N-M is the difficulty in deciding whether the onset of interference is a direct cause of the failure to produce N-M

in the sense that, double infection of single allantoic cells being prevented, no genetic interaction between the two strains is possible. For example, failure to isolate N-M may be explained quantitatively. If it is supposed that the ratio of N-M to MEL at the optimum dosage be constant at approximately $10^{2\cdot5}$ ID_{50} to $10^{7\cdot5}$ ID_{50}, a reduction of the yield of MEL to $10^{5\cdot0}$ ID_{50} will reduce the potential yield of N-M to very nearly *nil*. This objection to the direct influence of the establishment of interference cannot be sustained against the serological results. A merely quantitative explanation cannot explain the difference between the serological character of fluids in which N-M is found and that of fluids from which N-M is absent, and that change coincides with the onset of a measurable amount of interference. Under conditions of interference haemagglutinin behaves as though only one serological type or a simple mixture of both types were present. Before interference is established, the haemagglutinin behaves as if it were composed of aggregates of both serological types or else of particles possessing a double serological character.* This is strong evidence of mixed intracellular infection by NWS and MEL in which case we have to assume that interference prevents the appearance of such haemagglutinin by preventing double infection of single cells, although reproduction of both strains is still taking place in the allantoic cavity.

A point not established by the present data is whether a similar limiting time-interval exists with the reverse order of inoculation. Preliminary experiments using unwashed allantoic cavities, indicated that there was a very short time interval but this is not supported by the present findings. It would not be easy, for reasons already mentioned, to assess the amount of interference by MEL with the growth of NWS.

The fact that N-M is produced only when there is some degree of coincidence of the reproductive cycles of the two parent strains is itself likely evidence that one virus must influence the other by some genetic mechanism, even without the additional observation that interference and the production of recombinant strains are related phenomena.

SUMMARY.

In order to produce N-M (recombinant) virus in the course of one reproductive cycle of infection by NWS and MEL strains of Influenza A, the successive inoculations of NWS and MEL must be made within certain time limits.

The limiting time, when NWS precedes MEL, is not well defined, but is about two hours and coincides with the establishment of a measurable degree of interference by NWS.

The serological character of fluids containing N-M or of fluids obtained from those intervals of inoculation after which N-M is normally found, is distinct from the character of simple mixtures of NWS and MEL and from the character of the haemagglutinin present when some degree of interference is manifest.

For these reasons the conclusion is that interference acts directly in reducing the yield of N-M by preventing simultaneous reproduction of both parents in the same allantoic cell.

* Burnet (personal communication) believes that there is good evidence from other types of experiment that morphological units of virus may contain gene complexes corresponding to two different phenotypes. This may well be the case here.

The author is indebted to Sir Macfarlane Burnet, F.R.S., for valuable criticism of this article and for his kindness in introducing the author to this field of work, to Miss Patricia E. Lind for help in discussion and in the concluding experiments and to Miss Lois Larkin for very competent technical help.

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ON THE HISTOCHEMISTRY OF THE MYONEURAL JUNCTION.

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THE demonstration by a histochemical technique that an enzyme is active at a particular site is interesting in the promise it holds of ascribing function to microscopic structures. But this has been largely unfulfilled because the enzymes so localized have not been identified with the precision necessary to determine their function. Enzyme identification is the central problem in histochemistry and this paper describes the difficulties that are met when this problem is attacked. When the myoneural junctions of the rat diaphragm are shown up by the methods of Koelle (1950), and of Gomori (1948) for cholinesterase and of Nachlas and Seligman (1949*a*) for esterase it is apparent that the same structure is displayed by three methods using very different substrates. The problem is the definition in biochemical terms of the enzyme or enzymes responsible for these three pictures.

The fundamental limitation of histochemical methods lies in the choice of a substrate which must give on hydrolysis an insoluble product that can be converted into a coloured compound. A substrate that gives a good histological picture is usually so unnatural that the fact of its hydrolysis does not identify the enzyme responsible. Further, the known presence of an enzyme in a tissue does not necessarily mean that histochemical staining is due to this enzyme. The best histochemical staining is obtained where the enzyme is sharply localised and of high focal activity, and its overall activity in the tissue may be much less than that of a second enzyme distributed more generally. The difficulties raised by the presence of several enzymes similar in activity in the one tissue are notorious and this is the situation in the rat diaphragm. The enzymes are specific (or " true " or acetyl-) cholinesterase, non-specific (or " pseudo ") cholinesterase and ali-esterases (as defined by Richter and Croft, 1942) of which two, A- and B-esterase have been characterized by Aldridge (1953*a*). The problem can be illustrated by reference to the method of Nachlas and Seligman (1949*a*) for esterases in which β-naphthyl acetate is the substrate. Seligman and his colleagues have concluded that specific and non-specific cholinesterases as well as ali-esterases hydrolyse β-naphthyl acetate (Ravin, Tsou and Seligman, 1951; Barnnett and Seligman, 1951) and the staining may be due to any of these enzymes.

The method of Gomori (1948) is based on the hydrolysis of myristoyl choline. From the work of Adams and Whittaker (1948, 1949) it seems likely that myristoyl choline (containing a C_{14} acid radical) would be hydrolysed by non-specific rather

than by specific cholinesterase, and indeed Gomori found that two highly active preparations of specific cholinesterase did not hydrolyse myristoyl choline at all. These observations suggest nothing more than that the staining is due to an enzyme other than specific cholinesterase. The substrate for the method of Koelle (1950) is acetyl thiocholine and it is hydrolysed by both specific and non-specific cholinesterase. Koelle met this difficulty firstly by using DFP at a molar concentration of 10^{-6} to inhibit non-specific cholinesterase leaving the specific cholinesterase relatively unaffected, and secondly by using butyryl thiocholine as a selective substrate for non-specific cholinesterase. With these methods Koelle concluded that the skeletal muscle of the cat contains specific cholinesterase "almost exclusively." This conclusion is in conflict with the implications from the Gomori method and shows a need for an investigation of the specificity of the various methods.

In this paper these problems have been investigated in two ways.

1. The effect of selective inhibitors of specific and non-specific cholinesterase on histochemical staining has been studied. With each of the histochemical methods the concentration of inhibitor necessary to reduce staining to about half of normal intensity was found for the series of inhibitors and this pattern was compared with the known inhibitory powers of these compounds, against specific and non-specific cholinesterase, and as far as these are known, of aliesterase.

2. An attempt has been made to confirm the histological findings by biochemical means. The ability of active preparations of specific cholinesterase to hydrolyze myristoyl choline and β-naphthyl acetate has been studied. As the enzyme preparations are necessarily crude their effects may be due not to specific cholinesterase, but to other esterases present as contaminants. This point has been investigated by examining the effect of selective inhibitors on the activity of the enzyme preparations.

The present work was done as part of a study of the way in which organophosphorus compounds produce neuro-muscular block by inhibiting cholinesterase. The presence in muscle of several esterases capable of hydrolysing acetylcholine greatly complicates the study of events at the myoneural junctions in poisoning by anticholinesterases. This paper is concerned with the identification of these esterases and with their precise localization in the muscle of the rat diaphragm.

MATERIALS AND METHODS.

Rat diaphragm preparations.

The rats were killed by coal gas and the diaphragm removed in one piece by cutting close to the costal margin with scissors. The phrenic nerves sweep around the diaphragm from ventral to dorsal aspect and the myoneural junctions lie closely on either side of the nerves as they run at right angles to the long axis of the muscle fibres. If the excised diaphragm is held up to the light the course of the phrenic nerves can easily be seen. Pieces of diaphragm 6–10 mm. × 5 mm., were cut with scissors so that the phrenic nerve passed down the middle of the tissue which was then laid flat on the freezing head of the microtome. Frozen sections of the unfixed diaphragm were cut at 10–15μ. Each section contained a line of myoneural junctions, running down the middle of the section. The sections were floated out in water and were sufficiently robust to be manipulated with glass rods and passed through a series of solutions.

Many of the histological methods were modified so that frozen sections of fresh unfixed diaphragm could be used in place of the usual paraffin sections.

Gomori (1948) *method for cholinesterase.*

The prescribed buffer tris- (hydroxymethyl)-aminomethane was not obtainable in this country. A bicarbonate buffer was used instead. Fresh frozen sections were incubated for 30 min. in a mixture of 3 ml. 0·09 M sodium bicarbonate, 1 ml. 0·1 M cobalt acetate, 5 ml. distilled water and 1 ml. 0·02 M myristoyl choline. The mixture was gassed with 95 per cent N_2, 5 per cent CO_2 for 5 min. before use. After incubation the sections were washed in distilled water for 5 min., treated with dilute yellow ammonium sulphide, washed again, floated on to microscope slides, pressed flat with filter paper, passed through alcohol and xylol and mounted in Canada balsam. The substrate, myristoyl choline, was synthesised according to the method described by Gomori (1948).

Nachlas and Seligman (1949*a*) *method for esterase.*

This was modified by using Fast blue B (I.C.I.) instead of α-naphthyl diazonium naphthalene 1, 5-disulphonate as a coupling agent. Freshly cut frozen sections were placed in the substrate mixture at room temperature for 12 min., washed in distilled water and mounted on slides in glycerine jelly.

In the parallel experiments Gomori's (1950) modification was used in which α-naphthyl acetate is the substrate and the acetone is reduced to 0·5 ml. for each 10 mg. of substrate. Sections were placed in a freshly prepared and filtered solution of the substrate for 4 min. at room temperature. The sections were then washed in distilled water and mounted on slides in Farrant's medium.

Some diaphragms were fixed in cold acetone and embedded in paraffin according to the method of Nachlas and Seligman (1949*a*) and the sections were treated with the substrate as described in the original method.

Gomori (1945) *method for lipase.*

This method involving acetone fixation and paraffin embedding was followed for the diaphragm. The substrates were Tween 40 and 60. These are long chain fatty acid esters of substituted hexitans and hexides. In addition frozen sections of fresh diaphragm were incubated overnight in the substrate at 37°. They were washed in distilled water and mounted in glycerine jelly.

Koelle method for cholinesterase.

This method (Koelle and Friedenwald, 1949, Koelle, 1950) calls for fresh unfixed tissues. No improvement in intensity of staining or sharpness of localisation was obtained by saturating the substrate with copper thiocholine and this tedious step (Reagent 8, p. 164 in Koelle's 1950 paper) was omitted. A later modification including a salting-out technique (Koelle, 1951) gives greater precision of staining without altering the essential basis of the method. The main findings with the 1950 method have been confirmed with the 1951 method.

The acetyl thiocholine was synthesised in this laboratory by Mr. A. N. Davison by the method of Renshaw, Diesback, Ziff and Green (1938). The butyryl thiocholine was a gift from Dr. Bergel of Roche Products Ltd.

Inhibitors.

The following inhibitors were used :

mipafox	N N′-di-isopropyl phosphorodiamidic fluoride
paraoxon	diethyl *p*-nitrophenyl phosphate.
TEPP	tetraethyl pyrophosphate.
TIPP	tetra-isopropylpyrophosphate.
DFP	di-isopropyl fluorophosphonate.
iso-OMPA	tetra-isopropyl pyrophosphoramide.
eserine	(physostigmine).
NU 683	the dimethyl carbonate of (2-hydroxy-5 phenyl benzyl)-trimethyl ammonium bromide.
NU 1250	the N-*p*-chlorophenyl-N-methyl carbonate of *m*-hydroxy-phenyl trimethyl ammonium bromide.
284.c.51	1-5-bis (allyl dimethylammonium phenyl) pentane-3-ene dibromide.

With the irreversible inhibitors, paraoxon, TEPP, DFP, TIPP and iso-OMPA the effect on histochemical staining was studied by incubating sections without substrate at 37° in a series of dilutions of the inhibitor for exactly 30 min., removing and washing in three lots of 0·9 per cent NaCl and then transferring to the substrate used for the staining method. With the reversible inhibitors, eserine, NU 683, NU 1250 and 284.c.51, the sections were first placed in a solution of the inhibitor for 30 min. and then transferred to the substrate which also contained the inhibitor at the same concentration.

Warburg Experiments.

The cholinesterase activity was determined by the method of Nachmansohn and Rothenberg (1945) and the technical details were exactly those described by Aldridge (1950). Myristoyl choline was used at a final concentration of 0·01 M.

Defibrinated sheep red cells that had been stored at 4° were washed 3 times in isotonic saline and re-suspended in buffer to their original volume, immediately before use. Diaphragms were dissected whole from rats, weighed, homogenised in buffer and made up to a volume of 5 ml. or 10 ml. Aliquot parts of 1–5 ml. were taken for cholinesterase determinations. The brain of the rat excluding the cerebellum and medulla was also homogenised in buffer and made up to a final volume of 10 ml. Rat red cells obtained by cardiac puncture were citrated and treated similarly to sheep red cells.

Stroma was prepared by following the first part of the method given by Mounter and Whittaker (1950), 500 ml. of packed red cells from defibrinated sheep blood being lysed with 1,500 ml. of distilled water. The pH was adjusted to 6·5 with 0·1 N HCl. The preparation was centrifuged to collect the stroma and this was washed three times with distilled water. About 100 ml. of stroma was obtained in the form of a thin smooth paste that could still be pipetted and washed into reaction flasks. A purified preparation of specific cholinesterase from rat brain was made by the method of Ord and Thompson (1951).

Chemical Determination of Esterase Activity.

The method was a modification of that of Nachlas and Seligman (1949*b*). Fast blue B (I.C.I.) was used as the coupling agent. In experiments with paraoxon and DFP, 1 ml. of a 1 in 5 dilution of erythrocyte stroma was mixed in a 50 ml. tube with 1 ml. of an aqueous solution of the inhibitor to give the required final concentration of inhibitor, and the mixture was kept at 37° in a water bath. After 30 min. 6 ml. of the buffered solution of β-naphthyl acetate (as described by Nachlas and Seligman, 1949*b*) was added and was followed 16 min. later by 1 ml. of a freshly prepared solution of Fast blue B (2 mg./ml.). The colour was allowed to develop for 4 min. and then 1 ml. of 50 per cent trichloracetic acid was added and the tubes were removed from the water bath. The colour was extracted with 10 ml. of ethyl acetate, the solvent being separated by centrifuging at 1,000 r.p.m. for 5 min. The colour intensity was measured against a reagent blank in a Unicam spectrophotometer at a waveband of 535 Å.

As eserine is a competitive inhibitor and rapidly reaches an equilibrium, in experiments with eserine, the stroma, eserine and β-naphthyl acetate were added simultaneously. As eserine forms a coloured complex with the coupling agent Fast blue B it was necessary to control each concentration of eserine with a similar tube containing identical reagents with the exception of the stroma.

RESULTS.

Gomori method for cholinesterase.

The effect of inhibitors on staining by the Gomori method is shown in the Table. The concentration necessary to reduce the staining to about half intensity is compared with the concentrations necessary to produce 50 per cent inhibition of specific and of non-specific cholinesterase *in vitro*. The figures for 50 per cent inhibition are from many sources and some of the figures have been interpolated from data in the papers quoted. The results show that the staining is reduced

by concentrations of the various inhibitors very similar to the concentrations necessary to produce 50 per cent inhibition of specific cholinesterase. In particular the selective inhibitors of specific cholinesterase, NU 1250 (Hawkins and Mendel, 1949) and 284.c.51 (Austen and Berry, 1953) inhibit staining at very low concentrations. On the other hand the selective inhibitors of non-specific cholinesterase, NU 683 (Hawkins and Gunter, 1946), DFP (Ord and Thompson, 1950), iso-OMPA and mipafox (Aldridge, 1953*b*) prevent staining only when present at high concentrations relative to those required to produce 50 per cent inhibition of non-specific

TABLE.—*Comparison of the Concentrations of Anti-cholinesterases that will reduce Histochemical Staining to Half with the Concentrations producing 50 per cent Inhibition of Specific and Non-Specific Cholinesterase (determined by Warburg Technique).*

Inhibitor.	Molar concentration of inhibitor reducing intensity of staining to half. Method and substrate.				Molar concentration of inhibitor producing 50 per cent inhibition of enzyme.	
	Gomori	Nachlas and Seligman	Koelle.			
	Myristoyl choline.	β-naphthyl acetate.	Acetyl thio-choline.	Butyryl thio-choline.	Specific cholinesterase.	Non-specific cholinesterase.
TEPP	3×10^{-9}	10^{-8}	10^{-8}	10^{-9}	6×10^{-9} (*a*)	$8{\cdot}6 \times 10^{-10}$ (*h*)
Paraoxon	3×10^{-8}	3×10^{-8}	10^{-7}	3×10^{-8}	$2{\cdot}1 \times 10^{-8}$ (*b*)	$6{\cdot}5 \times 10^{-8}$ (*i*)
NU 683	3×10^{-8}	3×10^{-8}	10^{-7}	3×10^{-9}	10^{-8} (*c*)	2×10^{-9} (*c*)
DFP	10^{-7}	3×10^{-7}	10^{-6}	3×10^{-8}	$2{\cdot}7 \times 10^{-7}$ (*d*)	$8{\cdot}0 \times 10^{-9}$ (*d*)
Iso-OMPA	10^{-3}	10^{-3}	10^{-4}	3×10^{-7}	$3{\cdot}1 \times 10^{-3}$ (*e*)	$3{\cdot}3 \times 10^{-7}$ (*e*)
Mipafox	10^{-4}	10^{-4}	10^{-4}	3×10^{-7}	$4{\cdot}5 \times 10^{-5}$ (*e*)	$1{\cdot}7 \times 10^{-7}$ (*e*)
NU 1250	3×10^{-9}	10^{-8}	10^{-8}	3×10^{-5}	3×10^{-9} (*f*)	$6{\cdot}0 \times 10^{-5}$ (*f*)
284.c.51	3×10^{-6}	10^{-6}	10^{-6}	10^{-3}	5×10^{-6} (*g*)	10^{-3} (*g*)
Eserine	3×10^{-6}	10^{-7}	10^{-6}	3×10^{-7}	$1{\cdot}4 \times 10^{-6}$ (*b*)	4×10^{-8} (*j*)

Data for 50 per cent inhibition values from (*a*) Burgen, 1949; (*b*) Aldridge, 1950; (*c*) Hawkins and Gunter, 1946; (*d*) Ord and Thompson, 1950; (*e*) Aldridge, 1953*b*; (*f*) Hawkins and Mendel, 1949; (*g*) Austin and Berry, 1953; (*h*) Brauer, 1948; (*i*) Aldridge, personal communication, 1953; (*j*) Mackworth and Webb, 1948.

cholinesterase. Eserine is used as the only reliable method for separating cholinesterase from ali-esterases, cholinesterases being almost entirely inhibited by a molar concentration of 10^{-5}. At this concentration of eserine, staining is completely inhibited and so the staining cannot be attributed to the activity of an ali-esterase. The histochemical findings are consistent and suggest that the enzyme at the myoneural junction that hydrolyses myristoyl choline is specific cholinesterase.

In view of the histochemical findings it seems important to re-examine whether preparations of specific cholinesterase can hydrolyse myristoyl choline. Estimations of cholinesterase activity by the Warburg method show that appreciable hydrolysis of myristoyl choline is produced by highly active preparations of specific cholinesterase such as rat brain and sheep erythrocytes. There is the possibility, however, that this hydrolysis might be due to a second enzyme such as non-specific cholinesterase or an ali-esterase present as an impurity. This

point has been examined by determining the sensitivity to various inhibitors of the enzymic hydrolysis of acetyl choline and myristoyl choline by sheep erythrocyte stroma. If the two substrates behave similarly with two inhibitors, eserine and DFP, it would then be probable that the same enzyme hydrolyses the two substrates. The inhibition curves shown in Fig. 1 were obtained. With eserine, although the slopes of the inhibition curves are similar, there is, at all concentrations of inhibitor greater sensivitity to eserine inhibition when myristoyl choline is used than when acetyl choline is the substrate. This difference may be due to the fact that acetyl choline has a greater affinity than myristoyl choline for cholinesterase as judged by rates of hydrolysis. A reversible and competitive

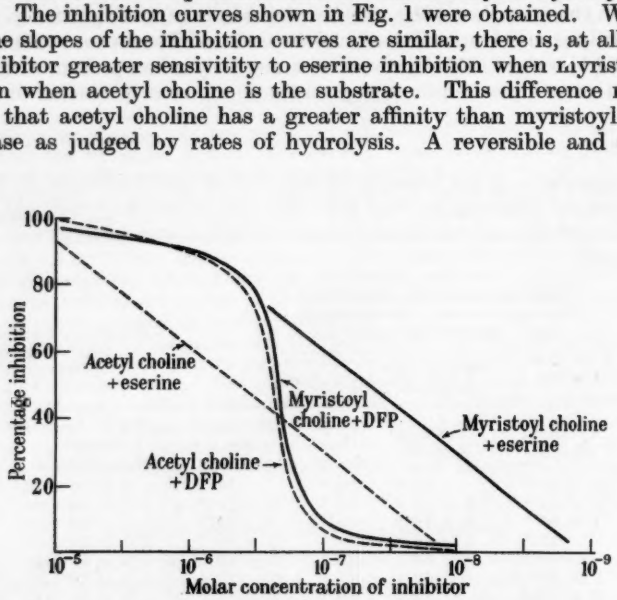


FIG. 1.—The effect of varying concentrations of the inhibitors eserine and DFP on the hydrolysis of myristoyl choline and acetyl choline by sheep erythrocyte stroma.

inhibitor such as eserine might be expected to compete for the enzyme more successfully against myristoyl choline than against acetyl choline. With DFP the inhibition curves are very similar for the two substrates.

Nachlas and Seligman method for esterase.

When fresh frozen sections are immersed in the Nachlas and Seligman substrate the muscle fibres stain uniformly crimson and the line of myoneural junctions stain purple. In paraffin sections of acetone-fixed tissues the general crimson staining persists but the localised purple staining at the myoneural junction is lost. With β-naphthyl acetate as substrate the staining at the myoneural junction takes the form of structureless blobs but with α-naphthyl acetate staining is much more precise (Fig. 4) and resembles closely that obtained with the Gomori method (Fig. 3) and the modified Koelle method (Fig. 5). The effect of various inhibitors of cholinesterase was the same with both α- and β-naphthyl acetate.

The Table shows that the pattern of inhibition of myoneural junction staining by the Nachlas and Seligman method is precisely that found with the Gomori method and corresponds to that of specific cholinesterase. The diffuse crimson staining is much more resistant to inhibition than the purple staining of the myoneural junctions. Thus the diffuse staining is scarcely affected by 10^{-3} M eserine or 10^{-5} M paraoxon. These findings suggest that the purple staining at the myoneural junctions is due to specific cholinesterase and the diffuse crimson staining to A-esterase.

This conclusion implies that specific cholinesterase will hydrolyse α- and β-naphthyl acetate and this point was examined by using the biochemical method of Nachlas and Seligman (1949*b*). With α-naphthyl acetate, the purple colour

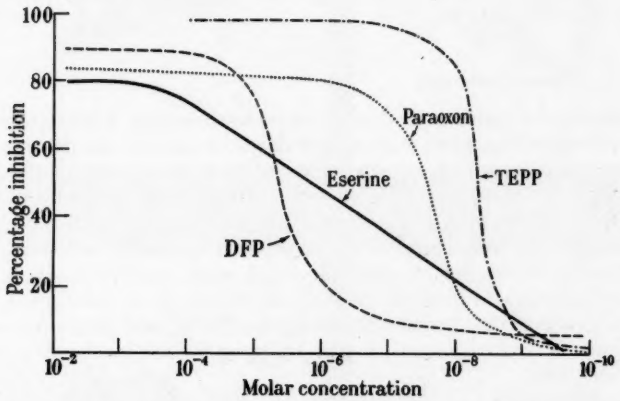


FIG. 2.—The effect of varying concentrations of the inhibitors eserine, DFP, TEPP and paraoxon on the hydrolysis of β-naphthyl acetate by rat brain cholinesterase. These inhibition curves are characteristic of specific cholinesterase: 50 per cent inhibition values are given by the following molar concentrations—eserine 10^{-6}, DFP 5×10^{-6}, TEPP 7×10^{-9}, paraoxon 4×10^{-8} (*cf.* column 5, Table).

that is formed absorbs strongly on to tissue fragments and is difficult to extract. For this reason β-naphthyl acetate was used in most of these experiments, but enough were done with α-naphthyl acetate to show that the results described for β-naphthyl acetate apply equally to α-naphthyl acetate.

Active but necessarily crude preparations of specific cholinesterase such as rat brain and diaphragm hydrolyse β-naphthyl acetate at rates similar to acetyl choline. Sheep erythrocyte stroma was found to contain an active and eserine-resistant ali-esterase that hydrolysed β-naphthyl acetate, interfering with the detection of any hydrolysis of this substrate by cholinesterase. Partially purified specific cholinesterase from rat brain prepared by the method of Ord and Thompson (1951) was free from non-specific cholinesterase and contained very little of either the acetate- or the butyrate-splitting ali-esterases (the A and B esterases differentiated by Aldridge, 1953*a*), while still retaining much of its specific cholin-

esterase activity. The effects of the inhibitors eserine, DFP, TEPP and paraoxon are shown in Fig. 2. About 80 per cent of the hydrolyses of β-naphthyl acetate is inhibited by each of the compounds at a concentration characteristic of its effect on specific cholinesterase (compare values of 50 per cent inhibition given in the Table). A small amount of the hydrolysis (about 20 per cent) is resistant to the effects of eserine, DFP and paraoxon and can probably be attributed to the small amount of A-esterase known to be present in the enzyme preparation.

Gomori method for lipase (esterase).

With fresh frozen sections, incubated for periods up to 8 hr., diffuse brown staining of muscle fibres was seen, but there was no focal staining at the myoneural junctions. The same results were obtained with acetone-fixed paraffin sections incubated in the substrate for 18 hr.

Koelle method for cholinesterase.

When acetyl thiocholine is used as a substrate the series of inhibitors produces a pattern of inhibition characteristic of their effect on specific cholinesterase (Table).

With butyryl thiocholine as substrate the results are very different. If the incubation time in the substrate is 30 min. as described in Koelle's (1950) paper no staining of the myoneural junctions is seen. If the incubation time is increased to 4 hr. clearly defined staining of the myoneural junctions is obtained (Fig. 6) and this staining is similar to that obtained with acetyl thiocholine (Fig. 5). The effect of selective inhibitors on the relatively slow hydrolysis of butyryl thiocholine is given in the fourth column of the Table, and it can be seen that the inhibition pattern is characteristic not of specific cholinesterase, but of non-specific cholinesterase.

DISCUSSION.

In the Gomori histochemical method, the enzyme hydrolysing the substrate myristoyl choline reacts with selective inhibitors in a way characteristic of specific cholinesterase. Active preparations of cholinesterase hydrolyse myristoyl choline at 3–5 per cent of the rate that they hydrolyse acetyl choline. Eserine was used to decide whether the hydrolysis of myristoyl choline by stroma was due to cholinesterase or an ali-esterase. Myristoyl choline hydrolysis by stroma proved to be more sensitive than acetyl choline hydrolysis to eserine inhibition. What-

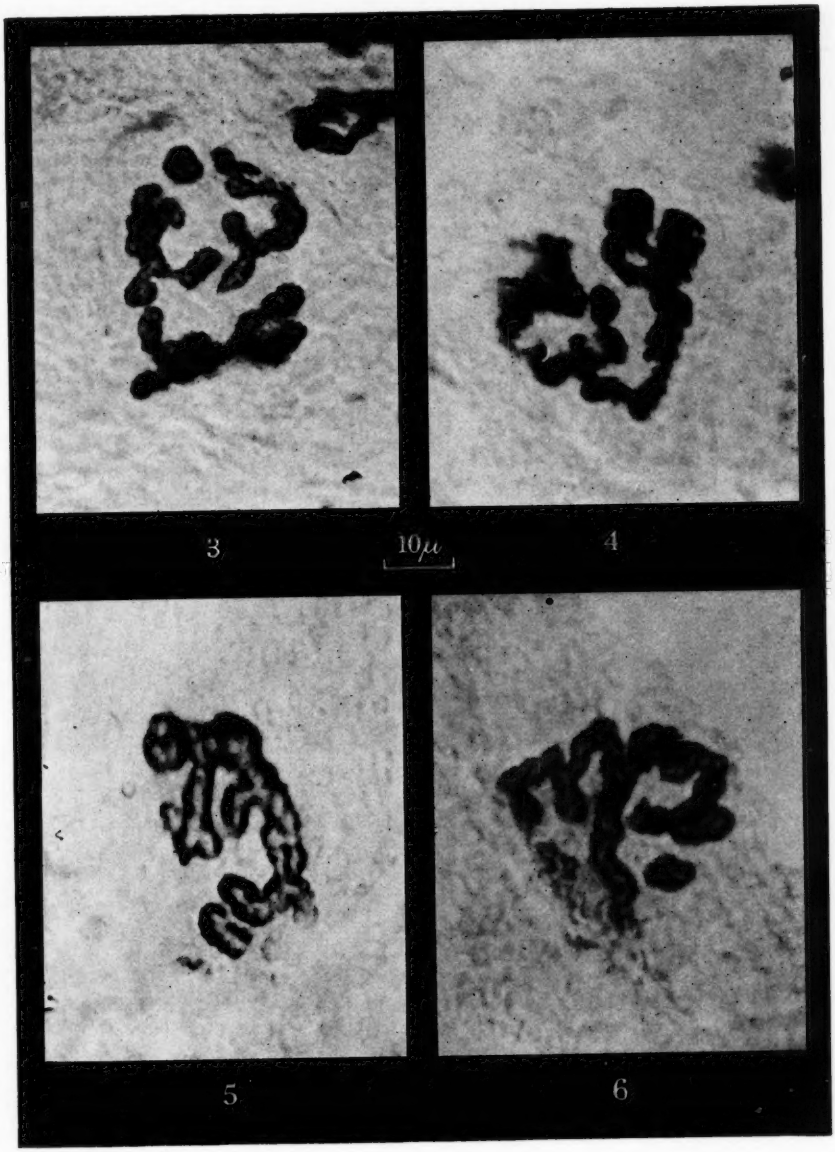
EXPLANATION OF PLATE.

FIG. 3.—Myoneural junction in rat diaphragm. Gomori (1948) method. Substrate—myristoyl choline.

FIG. 4.—Myoneural junction in rat diaphragm. Nachlas and Seligman (1949*a*) method. Substrate—α-naphthyl acetate.

FIG. 5.—Myoneural junction in rat diaphragm. Koelle (1951) method. Substrate—acetyl thiocholine.

FIG. 6.—Myoneural junction in rat diaphragm. Koelle (1951) method. Substrate—butyryl thiocholine.



Denz.

ever the explanation of the displacement of the two lines for eserine inhibition in Fig. 1 it is clear that the enzyme hydrolysing myristoyl choline is very sensitive to eserine inhibition. The curves for DFP are the same for the two substrates. The conclusion reached from these experiments is that specific cholinesterase can hydrolyse myristoyl choline and that the enzyme whose site of action is displayed in Fig. 3 is specific cholinesterase.

The Nachlas and Seligman method for esterases shows the presence of two enzymes in the diaphragm, the first highly active, sharply confined to the myoneural junctions and sensitive to inhibition by eserine and by selective inhibitors of specific cholinesterase and the second spread diffusely over the muscle fibre and resistant to inhibition by eserine. There is other evidence that these two enzymes differ in stability and other properties. If the diaphragm is fixed in cold acetone and paraffin-embedded according to the unmodified method of Nachlas and Seligman (1949*a*) the diffuse staining of the muscle fibres persists, but the myoneural staining is lost. The Gomori (1945) method for lipase shows up the sites of activity of ali-esterases (Nachlas and Seligman, 1949*a*) but there is no evidence that the substrates used in this method, the synthetic sorbitan esters of the higher fatty acids, can be hydrolysed by cholinesterase. With the Gomori method for lipase only diffuse staining of the muscles is observed without any evidence of activity of the myoneural junctions. These findings in conjunction with the effects of inhibitors on the staining by the modified Nachlas and Seligman method as summarised in the Table suggest that the enzyme at the myoneural junction that hydrolyses β-naphthyl acetate is specific cholinesterase and that the enzyme spread diffusely over the muscle fibre is an ali-esterase.

Evidence has been produced to show that specific cholinesterase obtained from rat brain will hydrolyse β-naphthyl acetate. This finding is in agreement with the conclusions of others that specific cholinesterase will hydrolyse a wide range or aromatic acetates (Whittaker, 1951). These observations confirm the histochemical finding that the staining at the myoneural junctions with the Nachlas and Seligman method (Fig. 4) is due to the activity of specific cholinesterase.

As the staining at the myoneural junctions by the Gomori method and the Nachlas and Seligman method can be accounted for by the activity of specific cholinesterase these methods do not disclose the presence of a second enzyme at the myoneural junction. On the other hand, the observation that butyryl thiocholine is hydrolysed at the myoneural junctions is itself suggestive that there is some non-specific cholinesterase activity since Koelle (1950) has shown that specific cholinesterase has little if any effect on butyryl thiocholine. Further, Davison (1953) has shown by using selective substrates and inhibitors that the whole diaphragm of the rat contains an appreciable amount of non-specific cholinesterase. The Gomori method and the Nachlas and Seligman method would not show up the presence of the relatively small activity of non-specific cholinesterase in the presence of considerable specific cholinesterase activity. An examination of the inhibition pattern of the enzyme hydrolysing butyryl thiocholine (Table) shows this to be characteristic of non-specific cholinesterase. An indication of the relative inactivity of the non-specific cholinesterase at the myoneural junction is obtained from the prolonged incubation necessary to obtain histochemical evidence of the activity of the enzyme. In tissues rich in non-specific cholinesterase such as salivary gland and ileum, 30 minutes is adequate for clear histochemical staining. At the myoneural junction staining is still somewhat pale

after the sections have been incubated with butyryl choline 8 times as long as the normal 30 minutes. This suggests that the non-specific cholinesterase at the myoneural junction posseses only a fraction of the hydrolytic activity shown by specific cholinesterase.

The provisional picture of the arrangement of these enzymes in the diaphragm can be drawn. Spread diffusely through the muscle there is an ali-esterase without any focal concentration. At the myoneural junction there are two enzymes sharply confined to the synaptic gutter. The specific cholinesterase is highly active and its site of action is displayed by the Koelle method for cholinesterase when acetyl thiocholine is used as a substrate and by the Gomori method for cholinesterase and by the Nachlas and Seligman method for esterase. The activity of the specific cholinesterase is so much greater than the activity of the non-specific cholinesterase that the latter is hidden with these methods. It has needed the increase in selectivity produced by the additive effects of a selective substrate, prolonged incubation and selective inhibitors to bring to light the non-specific cholinesterase at the myoneural junction.

SUMMARY.

Histochemical staining of the myoneural junctions of the rat diaphragm by the methods of Gomori, of Nachlas and Seligman, and of Koelle, when acetyl-thiocholine is the substrate, is due to the activity of specific cholinesterase.

Staining by the Koelle method with butyryl thiocholine as substrate is due to non-specific cholinesterase.

Specific cholinesterase is the main enzyme in the synaptic gutter at the myoneural junction, with a small amount of non-specific cholinesterase. An ali-esterase is spread diffusely throughout the muscle fibres without focal concentration at the myoneural junction.

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